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PREVENTION AND TREATMENT OF AMYLOIDOGENIC DISEASE

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PREVENTION AND TREATMENT OF AMYLOIDOGENIC DISEASE

CROSS-REFERENCES TO RELATED APPLICATIONS

[Handwritten: SLD]
This application is related to USSN 60/067,740, filed December 2, 1997, USSN 60/080,970, filed April 7, 1998, and USSN 09/201,430, filed November 30, 1998, each of which is incorporated by reference in its entirety for all purposes.

TECHNICAL FIELD

10 The invention resides in the technical fields of immunology and medicine.

BACKGROUND OF THE INVENTION

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Alzheimer's disease (AD) is a progressive disease resulting in senile dementia. *See generally* Selkoe, *TINS* 16, 403-409 (1993); Hardy et al., WO 92/13069; 15 Selkoe, *J. Neuropathol. Exp. Neurol.* 53, 438-447 (1994); Duff et al., *Nature* 373, 476-477 (1995); Games et al., *Nature* 373, 523 (1995). Broadly speaking the disease falls into two categories: late onset, which occurs in old age (65 + years) and early onset, which develops well before the senile period, i.e., between 35 and 60 years. In both types of disease, the pathology is the same but the abnormalities tend to be more severe and 20 widespread in cases beginning at an earlier age. The disease is characterized by at least two types of lesions in the brain, senile plaques and neurofibrillary tangles. Senile plaques are areas of disorganized neuropil up to 150 μ m across with extracellular amyloid deposits at the center visible by microscopic analysis of sections of brain tissue. Neurofibrillary tangles are intracellular deposits of microtubule associated tau protein 25 consisting of two filaments twisted about each other in pairs.

The principal constituent of the plaques is a peptide termed A β or β -amyloid peptide. A β peptide is an internal fragment of 39-43 amino acids of a precursor protein termed amyloid precursor protein (APP). Several mutations within the APP protein have been correlated with the presence of Alzheimer's disease. See, e.g., Goate et 30 al., *Nature* 349, 704 (1991) (valine⁷¹⁷ to isoleucine); Chartier Harlan et al. *Nature* 353, 844 (1991)) (valine⁷¹⁷ to glycine); Murrell et al., *Science* 254, 97 (1991) (valine⁷¹⁷ to phenylalanine); Mullan et al., *Nature Genet.* 1, 345 (1992) (a double mutation changing lysine⁵⁹⁵-methionine⁵⁹⁶ to asparagine⁵⁹⁵-leucine⁵⁹⁶). Such mutations are thought to cause

Alzheimer's disease by increased or altered processing of APP to A β , particularly processing of APP to increased amounts of the long form of A β (i.e., A β 1-42 and A β 1-43). Mutations in other genes, such as the presenilin genes, PS1 and PS2, are thought indirectly to affect processing of APP to generate increased amounts of long form A β (see Hardy, *TINS* 20, 154 (1997)). These observations indicate that A β , and particularly its long form, is a causative element in Alzheimer's disease.

McMichael, EP 526,511, proposes administration of homeopathic dosages (less than or equal to 10^{-2} mg/day) of A β to patients with preestablished AD. In a typical human with about 5 liters of plasma, even the upper limit of this dosage would be expected to generate a concentration of no more than 2 pg/ml. The normal concentration of A β in human plasma is typically in the range of 50-200 pg/ml (Seubert et al., *Nature* 359, 325-327 (1992)). Because EP 526,511's proposed dosage would barely alter the level of endogenous circulating A β and because EP 526,511 does not recommend use of an adjuvant, as an immunostimulant, it seems implausible that any therapeutic benefit would result.

By contrast, the present invention is directed *inter alia* to treatment of Alzheimer's and other amyloidogenic diseases by administration of A β , other active immunogen or antibody to A β to a patient under conditions that generate a beneficial immune response in the patient. The invention thus fulfills a longstanding need for therapeutic regimes for preventing or ameliorating the neuropathology and, in some patients, the cognitive impairment associated with Alzheimer's disease.

SUMMARY OF THE CLAIMED INVENTION

In one aspect, the invention provides methods of preventing or treating a disease characterized by amyloid deposit in a patient. Such methods entail administering an effective dosage of an antibody that specifically binds to the amyloid deposit or a component thereof to the patient. Such methods are particularly useful for preventing or treating Alzheimer's disease in which case the amyloid deposit is A β . The methods can be used on both asymptomatic patients and those currently showing symptoms of disease.

The antibody used in such methods can be a human, humanized, chimeric or nonhuman antibody and can be monoclonal or polyclonal. In some methods, the antibody is prepared from a human immunized with A β peptide, which human can be the patient to be treated with antibody.

In some methods, the antibody used binds to an epitope within residues 1-28 of A β . In some methods the antibody binds to an epitope within residues 1-10, and in some methods within residues 1-5. In some methods, the antibody specifically binds to A β peptide without binding to full-length amyloid precursor protein (APP).

5 In some methods antibody is administered at a dosage of at least 1 mg/kg body weight antibody. In some methods, the antibody is administered in multiple dosages over a period of at least six months. In some methods, the antibody is administered as a sustained release composition. The antibody can be administered, for example, intraperitoneally, orally, subcutaneously, intracranially, intramuscularly, topically or
10 intravenously.

In some methods, the antibody is administered by administering a polynucleotide encoding at least one antibody chain to the patient. The polynucleotide is expressed to produce the antibody chain in the patient. Optionally, the polynucleotide encodes heavy and light chains of the antibody. The polynucleotide is expressed to
15 produce the heavy and light chains in the patient.

In some methods, the patient is monitored for level of administered antibody in the blood of the patient.

In another aspect, the invention provides methods of preventing or treating Alzheimer's disease. These methods entail administering an effective dosage of a
20 polypeptide comprising an active fragment of A β that induces an immune response to A β in the patient. In some methods, the fragment comprises an epitope within amino acids 1-12 of A β . In some method, the fragment comprises an epitope within amino acids 1-16 of A β . In some methods, the fragment comprises an epitope within amino acids 13-28 of A β . In some methods, the fragment is free of at least the 5 C-terminal amino acids in
25 A β 43. In some methods, the fragment comprises up to 20 contiguous amino acids from A β . Fragments are typically administered at greater than 10 micrograms per dose per patient.

In some methods, the fragment is administered with an adjuvant that enhances the immune response to the A β peptide. The adjuvant and fragment can be
30 administered in either order or together as a composition. The adjuvant can be, for example, alum, MPL, QS-21 or incomplete Freund's adjuvant.

The invention further provides pharmaceutical compositions comprising active fragments of A β , such as described above, and an adjuvant.

The invention further provides methods of screening an antibody to A β or an active fragment of A β for use in treatment of Alzheimer's disease. Such methods entail administering an antibody that specifically binds to A β or a fragment of A β to a transgenic animal disposed to develop characteristics of Alzheimer's disease. One then
5 detects a reduction in the extent or rate of development of the characteristics relative to a control transgenic animal as a measure of the efficacy of the antibody or fragment. Optionally, antibodies can also be screened for capacity to bind an epitope within amino acids 1-28 or other epitope of A β .

10 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: Antibody titer after injection of transgenic mice with A β 1-42.

Fig. 2: Amyloid burden in the hippocampus. The percentage of the area of the hippocampal region occupied by amyloid plaques, defined by reactivity with the A β -specific monoclonal antibody 3D6, was determined by computer-assisted quantitative
15 image analysis of immunoreacted brain sections. The values for individual mice are shown sorted by treatment group. The horizontal line for each grouping indicates the median value of the distribution.

Fig 3: Neuritic dystrophy in the hippocampus. The percentage of the area of the hippocampal region occupied by dystrophic neurites, defined by their reactivity
20 with the human APP-specific monoclonal 8E5, was determined by quantitative computer-assisted image analysis of immunoreacted brain sections. The values for individual mice are shown for the AN1792-treated group and the PBS-treated control group. The horizontal line for each grouping indicates the median value of the distribution.

Fig. 4: Astrocytosis in the retrosplenial cortex. The percentage of the area
25 of the cortical region occupied by glial fibrillary acidic protein (GFAP)-positive astrocytes was determined by quantitative computer-assisted image analysis of immunoreacted brain sections. The values for individual mice are shown sorted by treatment group and median group values are indicated by horizontal lines.

Fig. 5: Geometric mean antibody titers to A β 1-42 following immunization
30 with a range of eight doses of AN1792 containing 0.14, 0.4, 1.2, 3.7, 11, 33, 100, or 300 μ g.

Fig. 6: Kinetics of antibody response to AN1792 immunization. Titers are expressed as geometric means of values for the 6 animals in each group.

Fig. 7: Quantitative image analysis of the cortical amyloid burden in PBS- and AN1792-treated mice.

Fig. 8: Quantitative image analysis of the neuritic plaque burden in PBS- and AN1792-treated mice.

5 Fig. 9: Quantitative image analysis of the percent of the retrosplenial cortex occupied by astrocytosis in PBS- and AN1792-treated mice.

Fig. 10: Lymphocyte Proliferation Assay on spleen cells from AN1792-treated (upper panel) or PBS-treated (lower panel).

10 Fig. 11: Total A β levels in the cortex. A scatterplot of individual A β profiles in mice immunized with A β or APP derivatives combined with Freund' adjuvant.

Fig. 12: Amyloid burden in the cortex was determined by quantitative image analysis of immunoreacted brain sections for mice immunized with the A β peptide conjugates A β 1-5, A β 1-12, and A β 13-28; the full length A β aggregates AN1792 (A β 1-42) and AN1528 (A β 1-40) and the PBS-treated control group.

15 Fig. 13: Geometric mean titers of A β -specific antibody for groups of mice immunized with A β or APP derivatives combined with Freund's adjuvant.

Fig. 14: Geometric mean titers of A β -specific antibody for groups of guinea pigs immunized with AN1792, or a palmitoylated derivative thereof, combined with various adjuvants.

20 *July 27* Fig. 15: A β levels in the cortex of 12-month old PDAPP mice treated with AN1792 or AN1528 with different adjuvants.

Fig. 16: Mean titer of mice treated with polyclonal antibody to A β .

Fig. 17: Mean titer of mice treated with monoclonal antibody 10D5 to A β .

Fig. 18: Mean titer of mice treated with monoclonal antibody 2F12 to A β .

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DEFINITIONS

The term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 65 percent sequence identity, preferably at least 80 or 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (*e.g.*, 99 percent sequence identity or higher). Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

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For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (*see generally* Ausubel *et al., supra*). One example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al., J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). Typically, default program parameters can be used to perform the sequence comparison, although customized parameters can also be used. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89, 10915 (1989))

For purposes of classifying amino acids substitutions as conservative or nonconservative, amino acids are grouped as follows: Group I (hydrophobic sidechains): norleucine, met, ala, val, leu, ile; Group II (neutral hydrophilic side chains): cys, ser, thr; Group III (acidic side chains): asp, glu; Group IV (basic side chains): asn, gln, his, lys, arg; Group V (residues influencing chain orientation): gly, pro; and Group VI (aromatic side chains): trp, tyr, phe. Conservative substitutions involve substitutions between amino acids in the same class. Non-conservative substitutions constitute exchanging a member of one of these classes for a member of another.

Therapeutic agents of the invention are typically substantially pure from undesired contaminant. This means that an agent is typically at least about 50% w/w (weight/weight) purity, as well as being substantially free from interfering proteins and

contaminants. Sometimes the agents are at least about 80% w/w and, more preferably at least 90 or about 95% w/w purity. However, using conventional protein purification techniques, homogeneous peptides of at least 99% w/w can be obtained.

Specific binding between two entities means an affinity of at least 10^6 , 10^7 , 10^8 , 10^9 M^{-1} , or 10^{10} M^{-1} . Affinities greater than 10^8 M^{-1} are preferred.

The term "antibody" or "immunoglobulin" is used to include intact antibodies and binding fragments thereof. Typically, fragments compete with the intact antibody from which they were derived for specific binding to an antigen fragments including separate heavy chains, light chains Fab, Fab' F(ab')₂, Fabc, and Fv. Fragments are produced by recombinant DNA techniques, or by enzymatic or chemical separation of intact immunoglobulins. The term "antibody" also includes one or more immunoglobulin chain that are chemically conjugated to, or expressed as, fusion proteins with other proteins. The term "antibody" also includes bispecific antibody. A bispecific or bifunctional antibody is an artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. See, e.g., Songsivilai & Lachmann, *Clin. Exp. Immunol.* 79:315-321 (1990); Kostelny et al., *J. Immunol.* 148, 1547-1553 (1992).

APP⁶⁹⁵, APP⁷⁵¹, and APP⁷⁷⁰ refer, respectively, to the 695, 751, and 770 amino acid residue long polypeptides encoded by the human APP gene. See Kang et al., *Nature* 325, 773 (1987); Ponte et al., *Nature* 331, 525 (1988); and Kitaguchi et al., *Nature* 331, 530 (1988). Amino acids within the human amyloid precursor protein (APP) are assigned numbers according to the sequence of the APP770 isoform. Terms such as A β 39, A β 40, A β 41, A β 42 and A β 43 refer to an A β peptide containing amino acid residues 1-39, 1-40, 1-41, 1-42 and 1-43.

The term "epitope" or "antigenic determinant" refers to a site on an antigen to which B and/or T cells respond. B-cell epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography

and 2-dimensional nuclear magnetic resonance. See, e.g., *Epitope Mapping Protocols in Methods in Molecular Biology*, Vol. 66, Glenn E. Morris, Ed. (1996). Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target antigen. T-cells recognize continuous epitopes of about nine amino acids for CD8 cells or about 13-15 amino acids for CD4 cells. T cells that recognize the epitope can be identified by *in vitro* assays that measure antigen-dependent proliferation, as determined by ³H-thymidine incorporation by primed T cells in response to an epitope (Burke et al., *J. Inf. Dis.* 170, 1110-19 (1994)), by antigen-dependent killing (cytotoxic T lymphocyte assay, Tigges et al., *J. Immunol.* 156, 3901-3910) or by cytokine secretion.

The term "immunological" or "immune" response is the development of a beneficial humoral (antibody mediated) and/or a cellular (mediated by antigen-specific T cells or their secretion products) response directed against an amyloid peptide in a recipient patient. Such a response can be an active response induced by administration of immunogen or a passive response induced by administration of antibody or primed T-cells. A cellular immune response is elicited by the presentation of polypeptide epitopes in association with Class I or Class II MHC molecules to activate antigen-specific CD4⁺ T helper cells and/or CD8⁺ cytotoxic T cells. The response may also involve activation of monocytes, macrophages, NK cells, basophils, dendritic cells, astrocytes, microglia cells, eosinophils or other components of innate immunity. The presence of a cell-mediated immunological response can be determined by proliferation assays (CD4⁺ T cells) or CTL (cytotoxic T lymphocyte) assays (see Burke, *supra*; Tigges, *supra*). The relative contributions of humoral and cellular responses to the protective or therapeutic effect of an immunogen can be distinguished by separately isolating antibodies and T-cells from an immunized syngeneic animal and measuring protective or therapeutic effect in a second subject.

An "immunogenic agent" or "immunogen" is capable of inducing an immunological response against itself on administration to a patient, optionally in conjunction with an adjuvant.

The term "naked polynucleotide" refers to a polynucleotide not complexed with colloidal materials. Naked polynucleotides are sometimes cloned in a plasmid vector.

The term "adjuvant" refers to a compound that when administered in conjunction with an antigen augments the immune response to the antigen, but when

administered alone does not generate an immune response to the antigen. Adjuvants can augment an immune response by several mechanisms including lymphocyte recruitment, stimulation of B and/or T cells, and stimulation of macrophages.

5 The term "patient" includes human and other mammalian subjects that receive either prophylactic or therapeutic treatment.

Disaggregated or monomeric A β means soluble, monomeric peptide units of A β . One method to prepare monomeric A β is to dissolve lyophilized peptide in neat DMSO with sonication. The resulting solution is centrifuged to remove any insoluble particulates. Aggregated A β is a mixture of oligomers in which the monomeric units are
10 held together by noncovalent bonds.

Competition between antibodies is determined by an assay in which the immunoglobulin under test inhibits specific binding of a reference antibody to a common antigen, such as A β . Numerous types of competitive binding assays are known, for example: solid phase direct or indirect radioimmunoassay (RIA), solid phase direct or
15 indirect enzyme immunoassay (EIA), sandwich competition assay (see Stahli et al., Methods in Enzymology 9:242-253 (1983)); solid phase direct biotin-avidin EIA (see Kirkland et al., J. Immunol. 137:3614-3619 (1986)); solid phase direct labeled assay, solid phase direct labeled sandwich assay (see Harlow and Lane, "Antibodies, A Laboratory Manual," Cold Spring Harbor Press (1988)); solid phase direct label RIA
20 using I-125 label (see Morel et al., Molec. Immunol. 25(1):7-15 (1988)); solid phase direct biotin-avidin EIA (Cheung et al., Virology 176:546-552 (1990)); and direct labeled RIA (Moldenhauer et al., Scand. J. Immunol. 32:77-82 (1990)). Typically, such an assay involves the use of purified antigen bound to a solid surface or cells bearing either of these, an unlabelled test immunoglobulin and a labelled reference immunoglobulin.

25 Competitive inhibition is measured by determining the amount of label bound to the solid surface or cells in the presence of the test immunoglobulin. Usually the test immunoglobulin is present in excess. Antibodies identified by competition assay (competing antibodies) include antibodies binding to the same epitope as the reference antibody and antibodies binding to an adjacent epitope sufficiently proximal to the
30 epitope bound by the reference antibody for steric hindrance to occur. Usually, when a competing antibody is present in excess, it will inhibit specific binding of a reference antibody to a common antigen by at least 50 or 75%.

Compositions or methods "comprising" one or more recited elements may include other elements not specifically recited. For example, a composition that comprises A β peptide encompasses both an isolated A β peptide and A β peptide as a component of a larger polypeptide sequence.

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DETAILED DESCRIPTION

I. General

The invention provides pharmaceutical compositions and methods for prophylactic and therapeutic treatment of diseases characterized by accumulation of amyloid deposits. Amyloid deposits comprise a peptide aggregated to an insoluble mass. The nature of the peptide varies in different diseases but in most cases, the aggregate has a β -pleated sheet structure and stains with Congo Red dye. Diseases characterized by amyloid deposits include Alzheimer's disease (AD), both late and early onset. In both diseases, the amyloid deposit comprises a peptide termed A β , which accumulates in the brain of affected individuals. Examples of some other diseases characterized by amyloid deposits are SAA amyloidosis, hereditary Icelandic syndrome, multiple myeloma, and spongiform encephalopathies, including mad cow disease, Creutzfeldt Jakob disease, sheep scrapie, and mink spongiform encephalopathy (see Weissmann et al., Curr. Opin. Neurobiol. 7, 695-700 (1997); Smits et al., Veterinary Quarterly 19, 101-105 (1997); Nathanson et al., Am. J. Epidemiol. 145, 959-969 (1997)). The peptides forming the aggregates in these diseases are serum amyloid A, cystatin C, IgG kappa light chain respectively for the first three, and prion protein for the others.

II. Therapeutic Agents

A. Alzheimer's Disease

1. Agents Inducing Active Immune Response

Therapeutic agents for use in the present invention induce an immune response against A β peptide. These agents include A β peptide itself and variants thereof, analogs and mimetics of A β peptide that induce and/or crossreact with antibodies to A β peptide, and antibodies or T-cells reactive with A β peptide. Induction of an immune response can be active as when an immunogen is administered to induce antibodies or T-cells reactive with A β in a patient, or passive, as when an antibody is administered that itself binds to A β in patient.

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A β , also known as β -amyloid peptide, or A4 peptide (see US 4,666,829; Glenner & Wong, Biochem. Biophys. Res. Commun. 120, 1131 (1984)), is a peptide of 39-43 amino acids, which is the principal component of characteristic plaques of Alzheimer's disease. A β is generated by processing of a larger protein APP by two enzymes, termed β and γ secretases (see Hardy, TINS 20, 154 (1997)). Known mutations in APP associated with Alzheimer's disease occur proximate to the site of β or γ secretase, or within A β . For example, position 717 is proximate to the site of γ -secretase cleavage of APP in its processing to A β , and positions 670/671 are proximate to the site of β -secretase cleavage. It is believed that the mutations cause AD by interacting with the cleavage reactions by which A β is formed so as to increase the amount of the 42/43 amino acid form of A β generated.

A β has the unusual property that it can fix and activate both classical and alternate complement cascades. In particular, it binds to Clq and ultimately to C3bi. This association facilitates binding to macrophages leading to activation of B cells. In addition, C3bi breaks down further and then binds to CR2 on B cells in a T cell dependent manner leading to a 10,000 increase in activation of these cells. This mechanism causes A β to generate an immune response in excess of that of other antigens.

The therapeutic agent used in the claimed methods can be any of the naturally occurring forms of A β peptide, and particularly the human forms (i.e., A β 39, A β 40, A β 41, A β 42 or A β 43). The sequences of these peptides and their relationship to the APP precursor are illustrated by Fig. 1 of Hardy et al., TINS 20, 155-158 (1997). For example, A β 42 has the sequence:

sub 1

H2N-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala-OH.

A β 41, A β 40 and A β 39 differ from A β 42 by the omission of Ala, Ala-Ile, and Ala-Ile-Val respectively from the C-terminal end. A β 43 differs from A β 42 by the presence of a threonine residue at the C-terminus. The therapeutic agent can also be an active fragment or analog of a natural A β peptide that contains an epitope that induces a similar protective or therapeutic immune response on administration to a human. Immunogenic fragments typically have a sequence of at least 3, 5, 6, 10 or 20 contiguous amino acids from a natural peptide. Immunogenic fragments include A β 1-5, 1-6, 1-10, 1-

12, 13-28, 17-28, 1-28, 25-35, 35-40 and 35-42. Fragments lacking at least one, and sometimes at least 5 or 10 C-terminal amino acid present in a naturally occurring forms of A β are used in some methods. For example, a fragment lacking 5 amino acids from the C-terminal end of AB43 includes the first 38 amino acids from the N-terminal end of AB.

- 5 Fragments from the N-terminal half of A β are preferred in some methods. Analogs include allelic, species and induced variants. Analogs typically differ from naturally occurring peptides at one or a few positions, often by virtue of conservative substitutions. Analogs typically exhibit at least 80 or 90% sequence identity with natural peptides. Some analogs also include unnatural amino acids or modifications of N or C terminal
- 10 amino acids. Examples of unnatural amino acids are α , α -disubstituted amino acids, N-alkyl amino acids, lactic acid, 4-hydroxyproline, γ -carboxyglutamate, ϵ -N,N,N-trimethyllysine, ϵ -N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω -N-methylarginine. Fragments and analogs can be screened for prophylactic or therapeutic efficacy in transgenic animal models in
- 15 comparison with untreated or placebo controls as described below.

A β , its fragments, analogs and other amyloidogenic peptides can be synthesized by solid phase peptide synthesis or recombinant expression, or can be obtained from natural sources. Automatic peptide synthesizers are commercially available from numerous suppliers, such as Applied Biosystems, Foster City, California.

20 Recombinant expression can be in bacteria, such as E. coli, yeast, insect cells or mammalian cells. Procedures for recombinant expression are described by Sambrook et al., *Molecular Cloning: A Laboratory Manual* (C.S.H.P. Press, NY 2d ed., 1989). Some forms of A β peptide are also available commercially (e.g., American Peptides Company, Inc., Sunnyvale, CA and California Peptide Research, Inc. Napa, CA).

- 25 Therapeutic agents also include longer polypeptides that include, for example, an A β peptide, active fragment or analog together with other amino acids. For example, A β peptide can be present as intact APP protein or a segment thereof, such as the C-100 fragment that begins at the N-terminus of A β and continues to the end of APP. Such polypeptides can be screened for prophylactic or therapeutic efficacy in animal
- 30 models in comparison with untreated or placebo controls as described below. The A β peptide, analog, active fragment or other polypeptide can be administered in associated form (i.e., as an β -amyloid peptide) or in dissociated form. Therapeutic agents also include multimers of monomeric immunogenic agents.

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In a further variation, an immunogenic peptide, such as A β , can be presented as a viral or bacterial vaccine. A nucleic acid encoding the immunogenic peptide is incorporated into a genome or episome of the virus or bacteria. Optionally, the nucleic acid is incorporated in such a manner that the immunogenic peptide is expressed as a secreted protein or as a fusion protein with an outersurface protein of a virus or a transmembrane protein of a bacteria so that the peptide is displayed. Viruses or bacteria used in such methods should be nonpathogenic or attenuated. Suitable viruses include adenovirus, HSV, vaccinia and fowl pox. Fusion of an immunogenic peptide to HBsAg of HBV is particularly suitable. Therapeutic agents also include peptides and other compounds that do not necessarily have a significant amino acid sequence similarity with A β but nevertheless serve as mimetics of A β and induce a similar immune response. For example, any peptides and proteins forming β -pleated sheets can be screened for suitability. Anti-idiotypic antibodies against monoclonal antibodies to A β or other amyloidogenic peptides can also be used. Such anti-Id antibodies mimic the antigen and generate an immune response to it (see *Essential Immunology* (Roit ed., Blackwell Scientific Publications, Palo Alto, 6th ed.), p. 181).

Random libraries of peptides or other compounds can also be screened for suitability. Combinatorial libraries can be produced for many types of compounds that can be synthesized in a step-by-step fashion. Such compounds include polypeptides, beta-turn mimetics, polysaccharides, phospholipids, hormones, prostaglandins, steroids, aromatic compounds, heterocyclic compounds, benzodiazepines, oligomeric N-substituted glycines and oligocarbamates. Large combinatorial libraries of the compounds can be constructed by the encoded synthetic libraries (ESL) method described in Affymax, WO 95/12608, Affymax, WO 93/06121, Columbia University, WO 94/08051, Pharmacocea, WO 95/35503 and Scripps, WO 95/30642 (each of which is incorporated by reference for all purposes). Peptide libraries can also be generated by phage display methods. See, e.g., Devlin, WO 91/18980.

Combinatorial libraries and other compounds are initially screened for suitability by determining their capacity to bind to antibodies or lymphocytes (B or T) known to be specific for A β or other amyloidogenic peptides. For example, initial screens can be performed with any polyclonal sera or monoclonal antibody to A β or other amyloidogenic peptide. Compounds identified by such screens are then further analyzed for capacity to induce antibodies or reactive lymphocytes to A β or other amyloidogenic

peptide. For example, multiple dilutions of sera can be tested on microtiter plates that have been precoated with A β peptide and a standard ELISA can be performed to test for reactive antibodies to A β . Compounds can then be tested for prophylactic and therapeutic efficacy in transgenic animals predisposed to an amyloidogenic disease, as described in the Examples. Such animals include, for example, mice bearing a 717 mutation of APP described by Games et al., supra, and mice bearing a Swedish mutation of APP such as described by McConlogue et al., US 5,612,486 and Hsiao et al., *Science* 274, 99 (1996); Staufenbiel et al., *Proc. Natl. Acad. Sci. USA* 94, 13287-13292 (1997); Sturchler-Pierrat et al., *Proc. Natl. Acad. Sci. USA* 94, 13287-13292 (1997); Borchelt et al., *Neuron* 19, 939-945 (1997)). The same screening approach can be used on other potential agents such as fragments of A β , analogs of A β and longer peptides including A β , described above.

2. Agents Inducing Passive Immune Response

Therapeutic agents of the invention also include antibodies that specifically bind to A β or other component of amyloid plaques. Such antibodies can be monoclonal or polyclonal. Some such antibodies bind specifically to the aggregated form of A β without binding to the dissociated form. Some bind specifically to the dissociated form without binding to the aggregated form. Some bind to both aggregated and dissociated forms. Some such antibodies bind to a naturally occurring short form of A β (i.e., A β 39, 40 or 41) without binding to a naturally occurring long form of A β (i.e., A β 42 and A β 43). Some antibodies bind to a long form without binding to a short form. Some antibodies bind to A β without binding to full-length amyloid precursor protein. Some antibodies bind to A β with a binding affinity greater than or equal to about 10^6 , 10^7 , 10^8 , 10^9 , or 10^{10} M $^{-1}$.

25 *See c1* Polyclonal sera typically contain mixed populations of antibodies binding to several epitopes along the length of A β . Monoclonal antibodies bind to a specific epitope within A β that can be a conformational or nonconformational epitope. Some monoclonal antibodies bind to an epitope within residues 1-28 of A β (with the first N terminal residue of natural A β designated 1). Some monoclonal antibodies bind to an epitope with residues 1-10 of A β . Some monoclonal antibodies bind to an epitope with residues 1-16 of A β . Some monoclonal antibodies bind to an epitope with residues 1-25 of A β . Some monoclonal antibodies bind to an epitope within amino acids 1-5, 5-10, 10-

15, 15-20, 25-30, 10-20, 20, 30, or 10-25 of A β . Prophylactic and therapeutic efficacy of antibodies can be tested using the transgenic animal model procedures described in the Examples.

5 i. General Characteristics of Immunoglobulins

The basic antibody structural unit is known to comprise a tetramer of subunits. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or
10 more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function.

Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable
15 and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. (*See generally, Fundamental Immunology* (Paul, W., ed., 2nd ed. Raven Press, N.Y., 1989), Ch. 7 (incorporated by reference in its entirety for all purposes).

The variable regions of each light/heavy chain pair form the antibody
20 binding site. Thus, an intact antibody has two binding sites. Except in bifunctional or bispecific antibodies, the two binding sites are the same. The chains all exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarity determining regions or CDRs. The CDRs from the two chains of each pair are aligned by the framework regions, enabling
25 binding to a specific epitope. From N-terminal to C-terminal, both light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is in accordance with the definitions of Kabat, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987 and 1991), or Chothia & Lesk, *J. Mol. Biol.* 196:901-917 (1987); Chothia et al., *Nature*
30 342:878-883 (1989).

ii. Production of Nonhuman Antibodies

The production of non-human monoclonal antibodies, e.g., murine, guinea pig, rabbit or rat, can be accomplished by, for example, immunizing the animal with A β . A longer polypeptide comprising A β or an immunogenic fragment of A β or anti-idiotypic antibodies to an antibody to A β . See Harlow & Lane, *Antibodies, A Laboratory Manual* (CSHP NY, 1988) (incorporated by reference for all purposes). Such an immunogen can be obtained from a natural source, by peptide synthesis or by recombinant expression. Optionally, the immunogen can be administered fused or otherwise complexed with a carrier protein, as described below. Optionally, the immunogen can be administered with an adjuvant. Several types of adjuvant can be used as described below. Complete Freund's adjuvant followed by incomplete adjuvant is preferred for immunization of laboratory animals. Rabbits or guinea pigs are typically used for making polyclonal antibodies. Mice are typically used for making monoclonal antibodies. Antibodies are screened for specific binding to A β . Optionally, antibodies are further screened for binding to a specific region of A β . The latter screening can be accomplished by determining binding of an antibody to a collection of deletion mutants of an A β peptide and determining which deletion mutants bind to the antibody. Binding can be assessed, for example by Western blot or ELISA. The smallest fragment to show specific binding to the antibody defines the epitope of the antibody. Alternatively, epitope specificity can be determined by a competition assay in which a test and reference antibody compete for binding to A β . If the test and reference antibodies compete, then they bind to the same epitope or epitopes sufficiently proximal that binding of one antibody interferes with binding of the other.

iii. Chimeric and Humanized Antibodies

Chimeric and humanized antibodies have the same or similar binding specificity and affinity as a mouse or other nonhuman antibody that provides the starting material for construction of a chimeric or humanized antibody. Chimeric antibodies are antibodies whose light and heavy chain genes have been constructed, typically by genetic engineering, from immunoglobulin gene segments belonging to different species. For example, the variable (V) segments of the genes from a mouse monoclonal antibody may be joined to human constant (C) segments, such as IgG1 and IgG4. A typical chimeric

antibody is thus a hybrid protein consisting of the V or antigen-binding domain from a mouse antibody and the C or effector domain from a human antibody.

Humanized antibodies have variable region framework residues substantially from a human antibody (termed an acceptor antibody) and complementarity determining regions substantially from a mouse-antibody, (referred to as the donor immunoglobulin). See, Queen et al., *Proc. Natl. Acad. Sci. USA* 86:10029-10033 (1989) and WO 90/07861, US 5,693,762, US 5,693,761, US 5,585,089, US 5,530,101 and Winter, US 5,225,539 (incorporated by reference in their entirety for all purposes). The constant region(s), if present, are also substantially or entirely from a human immunoglobulin. The human variable domains are usually chosen from human antibodies whose framework sequences exhibit a high degree of sequence identity with the murine variable region domains from which the CDRs were derived. The heavy and light chain variable region framework residues can be derived from the same or different human antibody sequences. The human antibody sequences can be the sequences of naturally occurring human antibodies or can be consensus sequences of several human antibodies. See Carter et al., WO 92/22653. Certain amino acids from the human variable region framework residues are selected for substitution based on their possible influence on CDR conformation and/or binding to antigen. Investigation of such possible influences is by modeling, examination of the characteristics of the amino acids at particular locations, or empirical observation of the effects of substitution or mutagenesis of particular amino acids.

For example, when an amino acid differs between a murine variable region framework residue and a selected human variable region framework residue, the human framework amino acid should usually be substituted by the equivalent framework amino acid from the mouse antibody when it is reasonably expected that the amino acid:

- (1) noncovalently binds antigen directly,
- (2) is adjacent to a CDR region,
- (3) otherwise interacts with a CDR region (e.g. is within about 6 Å of a CDR region), or
- (4) participates in the VL-VH interface.

Other candidates for substitution are acceptor human framework amino acids that are unusual for a human immunoglobulin at that position. These amino acids can be substituted with amino acids from the equivalent position of the mouse donor antibody or from the equivalent positions of more typical human immunoglobulins. Other

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candidates for substitution are acceptor human framework amino acids that are unusual for a human immunoglobulin at that position.. The variable region frameworks of humanized immunoglobulins usually show at least 85% sequence identity to a human variable region framework sequence or consensus of such sequences.

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iv. Human Antibodies

Human antibodies against A β are provided by a variety of techniques described below. Some human antibodies are selected by competitive binding experiments, or otherwise, to have the same epitope specificity as a particular mouse antibody, such as one of the mouse monoclonals described in Example XI. Human antibodies can also be screened for a particular epitope specificity by using only a fragment of A β as the immunogen, and/or by screening antibodies against a collection of deletion mutants of A β .

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(1) Trioma Methodology

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The basic approach and an exemplary cell fusion partner, SPAZ-4, for use in this approach have been described by Oestberg et al., *Hybridoma* 2:361-367 (1983); Oestberg, U.S. Patent No. 4,634,664; and Engleman et al., US Patent 4,634,666 (each of which is incorporated by reference in its entirety for all purposes). The antibody-producing cell lines obtained by this method are called triomas, because they are descended from three cells--two human and one mouse. Initially, a mouse myeloma line is fused with a human B-lymphocyte to obtain a non-antibody-producing xenogeneic hybrid cell, such as the SPAZ-4 cell line described by Oestberg, supra. The xenogeneic cell is then fused with an immunized human B-lymphocyte to obtain an antibody-producing trioma cell line. Triomas have been found to produce antibody more stably than ordinary hybridomas made from human cells.

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The immunized B-lymphocytes are obtained from the blood, spleen, lymph nodes or bone marrow of a human donor. If antibodies against a specific antigen or epitope are desired, it is preferable to use that antigen or epitope thereof for immunization. Immunization can be either in vivo or in vitro. For in vivo immunization, B cells are typically isolated from a human immunized with A β , a fragment thereof, larger polypeptide containing A β or fragment, or an anti-idiotypic antibody to an antibody to A β . In some methods, B cells are isolated from the same patient who is ultimately to be administered antibody therapy.- For in vitro immunization, B-

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lymphocytes are typically exposed to antigen for a period of 7-14 days in a media such as RPMI-1640 (see Engleman, *supra*) supplemented with 10% human plasma.

The immunized B-lymphocytes are fused to a xenogeneic hybrid cell such as SPAZ-4 by well known methods. For example, the cells are treated with 40-50% polyethylene glycol of MW 1000-4000, at about 37 degrees, for about 5-10 min. Cells are separated from the fusion mixture and propagated in media selective for the desired hybrids (e.g., HAT or AH). Clones secreting antibodies having the required binding specificity are identified by assaying the trioma culture medium for the ability to bind to A β or a fragment thereof. Triomas producing human antibodies having the desired specificity are subcloned by the limiting dilution technique and grown in vitro in culture medium. The trioma cell lines obtained are then tested for the ability to bind A β or a fragment thereof.

Although triomas are genetically stable they do not produce antibodies at very high levels. Expression levels can be increased by cloning antibody genes from the trioma into one or more expression vectors, and transforming the vector into standard mammalian, bacterial or yeast cell lines.

(2) Transgenic Non-Human Mammals

Human antibodies against A β can also be produced from non-human transgenic mammals having transgenes encoding at least a segment of the human immunoglobulin locus. Usually, the endogenous immunoglobulin locus of such transgenic mammals is functionally inactivated. Preferably, the segment of the human immunoglobulin locus includes unrearranged sequences of heavy and light chain components. Both inactivation of endogenous immunoglobulin genes and introduction of exogenous immunoglobulin genes can be achieved by targeted homologous recombination, or by introduction of YAC chromosomes. The transgenic mammals resulting from this process are capable of functionally rearranging the immunoglobulin component sequences, and expressing a repertoire of antibodies of various isotypes encoded by human immunoglobulin genes, without expressing endogenous immunoglobulin genes. The production and properties of mammals having these properties are described in detail by, e.g., Lonberg et al., WO93/12227 (1993); US 5,877,397, US 5,874,299, US 5,814,318, US 5,789,650, US 5,770,429, US 5,661,016, US 5,633,425, US 5,625,126, US 5,569,825, US 5,545,806, *Nature* 148, 1547-1553 (1994),

Nature Biotechnology 14, 826 (1996), Kucherlapati, WO 91/10741 (1991) (each of which is incorporated by reference in its entirety for all purposes). Transgenic mice are particularly suitable. Anti-A β antibodies are obtained by immunizing a transgenic nonhuman mammal, such as described by Lonberg or Kucherlapati, *supra*, with A β or a fragment thereof. Monoclonal antibodies are prepared by, e.g., fusing B-cells from such mammals to suitable myeloma cell lines using conventional Kohler-Milstein technology. Human polyclonal antibodies can also be provided in the form of serum from humans immunized with an immunogenic agent. Optionally, such polyclonal antibodies can be concentrated by affinity purification using A β or other amyloid peptide as an affinity reagent.

(3) Phage Display Methods

A further approach for obtaining human anti-A β antibodies is to screen a DNA library from human B cells according to the general protocol outlined by Huse et al., *Science* 246:1275-1281 (1989). As described for trioma methodology, such B cells can be obtained from a human immunized with A β , fragments, longer polypeptides containing A β or fragments or anti-idiotypic antibodies. Optionally, such B cells are obtained from a patient who is ultimately to receive antibody treatment. Antibodies binding to A β or a fragment thereof are selected. Sequences encoding such antibodies (or a binding fragments) are then cloned and amplified. The protocol described by Huse is rendered more efficient in combination with phage-display technology. See, e.g., Dower et al., WO 91/17271 and McCafferty et al., WO 92/01047, US 5,877,218, US 5,871,907, US 5,858,657, US 5,837,242, US 5,733,743 and US 5,565,332 (each of which is incorporated by reference in its entirety for all purposes). In these methods, libraries of phage are produced in which members display different antibodies on their outer surfaces. Antibodies are usually displayed as Fv or Fab fragments. Phage displaying antibodies with a desired specificity are selected by affinity enrichment to an A β peptide or fragment thereof.

In a variation of the phage-display method, human antibodies having the binding specificity of a selected murine antibody can be produced. See Winter, WO 92/20791. In this method, either the heavy or light chain variable region of the selected murine antibody is used as a starting material. If, for example, a light chain variable region is selected as the starting material, a phage library is constructed in which

members display the same light chain variable region (i.e., the murine starting material) and a different heavy chain variable region. The heavy chain variable regions are obtained from a library of rearranged human heavy chain variable regions. A phage showing strong specific binding for A β (e.g., at least 10^8 and preferably at least 10^9 M $^{-1}$) is selected. The human heavy chain variable region from this phage then serves as a starting material for constructing a further phage library. In this library, each phage displays the same heavy chain variable region (i.e., the region identified from the first display library) and a different light chain variable region. The light chain variable regions are obtained from a library of rearranged human variable light chain regions. Again, phage showing strong specific binding for A β are selected. These phage display the variable regions of completely human anti-A β antibodies. These antibodies usually have the same or similar epitope specificity as the murine starting material.

v. Selection of Constant Region

The heavy and light chain variable regions of chimeric, humanized, or human antibodies can be linked to at least a portion of a human constant region. The choice of constant region depends, in part, whether antibody-dependent complement and/or cellular mediated toxicity is desired. For example, isotopes IgG1 and IgG3 have complement activity and isotypes IgG2 and IgG4 do not. Choice of isotype can also affect passage of antibody into the brain. Light chain constant regions can be lambda or kappa. Antibodies can be expressed as tetramers containing two light and two heavy chains, as separate heavy chains, light chains, as Fab, Fab' F(ab') $_2$, and Fv, or as single chain antibodies in which heavy and light chain variable domains are linked through a spacer.

vi. Expression of Recombinant Antibodies

Chimeric, humanized and human antibodies are typically produced by recombinant expression. Recombinant polynucleotide constructs typically include an expression control sequence operably linked to the coding sequences of antibody chains, including naturally-associated or heterologous promoter regions. Preferably, the expression control sequences are eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level

expression of the nucleotide sequences, and the collection and purification of the crossreacting antibodies.

These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors contain selection markers, e.g., ampicillin-resistance or hygromycin-resistance, to permit detection of those cells transformed with the desired DNA sequences.

E. coli is one prokaryotic host particularly useful for cloning the DNA sequences of the present invention. Microbes, such as yeast are also useful for expression. *Saccharomyces* is a preferred yeast host, with suitable vectors having expression control sequences, an origin of replication, termination sequences and the like as desired. Typical promoters include 3-phosphoglycerate kinase and other glycolytic enzymes. Inducible yeast promoters include, among others, promoters from alcohol dehydrogenase, isocytochrome C, and enzymes responsible for maltose and galactose utilization.

Mammalian cells are a preferred host for expressing nucleotide segments encoding immunoglobulins or fragments thereof. See Winnacker, *From Genes to Clones*, (VCH Publishers, NY, 1987). A number of suitable host cell lines capable of secreting intact heterologous proteins have been developed in the art, and include CHO cell lines, various COS cell lines, HeLa cells, L cells and myeloma cell lines. Preferably, the cells are nonhuman. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen et al., *Immunol. Rev.* 89:49 (1986)), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from endogenous genes, cytomegalovirus, SV40, adenovirus, bovine papillomavirus, and the like. See Co et al., *J. Immunol.* 148:1149 (1992).

Alternatively, antibody coding sequences can be incorporated in transgenes for introduction into the genome of a transgenic animal and subsequent expression in the milk of the transgenic animal (see, e.g., US 5,741,957, US 5,304,489, US 5,849,992). Suitable transgenes include coding sequences for light and/or heavy chains in operable linkage with a promoter and enhancer from a mammary gland specific gene, such as casein or beta lactoglobulin.

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The vectors containing the DNA segments of interest can be transferred into the host cell by well-known methods, depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment, electroporation, lipofection, biolistics or viral-based transfection can be used for other cellular hosts. Other methods used to transform mammalian cells include the use of polybrene, protoplast fusion, liposomes, electroporation, and microinjection (see generally, Sambrook et al., supra). For production of transgenic animals, transgenes can be microinjected into fertilized oocytes, or can be incorporated into the genome of embryonic stem cells, and the nuclei of such cells transferred into enucleated oocytes.

Once expressed, antibodies can be purified according to standard procedures of the art, including HPLC purification, column chromatography, gel electrophoresis and the like (see generally, Scopes, *Protein Purification* (Springer-Verlag, NY, 1982)).

4. Other Therapeutic Agents

Therapeutic agents for use in the present methods also include T-cells that bind to A β peptide. For example, T-cells can be activated against A β peptide by expressing a human MHC class I gene and a human β -2-microglobulin gene from an insect cell line, whereby an empty complex is formed on the surface of the cells and can bind to A β peptide. T-cells contacted with the cell line become specifically activated against the peptide. See Peterson et al., US 5,314,813. Insect cell lines expressing an MHC class II antigen can similarly be used to activate CD4 T cells.

B. Other Diseases

The same or analogous principles determine production of therapeutic or preventative agents for amyloidogenic diseases. In general, the agents noted above for use in treatment of Alzheimer's disease can also be used for treatment early onset Alzheimer's disease associated with Down's syndrome. In mad cow disease, prion peptide, active fragments, and analogs, and antibodies to prion peptide are used in place of A β peptide, active fragments, analogs and antibodies to A β peptide in treatment of Alzheimer's disease. In treatment of multiple myeloma, IgG light chain and analogs and antibodies thereto are used, and so forth in other diseases.

1. Carrier Proteins

Some agents for inducing an immune response contain the appropriate epitope for inducing an immune response against amyloid deposits but are too small to be immunogenic. In this situation, a peptide immunogen can be linked to a suitable carrier to help elicit an immune response. Suitable carriers include serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, or a toxoid from other pathogenic bacteria, such as diphtheria, *E. coli*, cholera, or *H. pylori*, or an attenuated toxin derivative. Other carriers for stimulating or enhancing an immune response include cytokines such as IL-1, IL-1 α and β peptides, IL-2, Γ TNF, IL-10, GM-CSF, and chemokines, such as MIP1 α and β and RANTES. Immunogenic agents can also be linked to peptides that enhance transport across tissues, as described in O'Mahony, WO 97/17613 and WO 97/17614.

Immunogenic agents can be linked to carriers by chemical crosslinking.

Techniques for linking an immunogen to a carrier include the formation of disulfide linkages using N-succinimidyl-3-(2-pyridyl-thio) propionate (SPDP) and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) (if the peptide lacks a sulfhydryl group, this can be provided by addition of a cysteine residue). These reagents create a disulfide linkage between themselves and peptide cysteine residues on one protein and an amide linkage through the α -amino on a lysine, or other free amino group in other amino acids. A variety of such disulfide/amide-forming agents are described by *Immun. Rev.* 62, 185 (1982). Other bifunctional coupling agents form a thioether rather than a disulfide linkage. Many of these thio-ether-forming agents are commercially available and include reactive esters of 6-maleimidocaproic acid, 2-bromoacetic acid, and 2-iodoacetic acid, 4-(N-maleimido-methyl)cyclohexane-1-carboxylic acid. The carboxyl groups can be activated by combining them with succinimide or 1-hydroxyl-2-nitro-4-sulfonic acid, sodium salt.

Immunogenic peptides can also be expressed as fusion proteins with carriers. The immunogenic peptide can be linked at the amino terminus, the carboxyl terminus, or internally to the carrier. Optionally, multiple repeats of the immunogenic peptide can be present in the fusion protein.

The same or similar carrier proteins and methods of linkage can be used for generating immunogens to be used in generation of antibodies against A β for use in

passive immunization. For example, A β or a fragment linked to a carrier can be administered to a laboratory animal in the production of monoclonal antibodies to A β .

4. Nucleic Acid Encoding Therapeutic Agents

Immune responses against amyloid deposits can also be induced by administration of nucleic acids encoding A β peptide, other peptide immunogens, or antibodies and their component chains used for passive immunization. Such nucleic acids can be DNA or RNA. A nucleic acid segment encoding an immunogen is typically linked to regulatory elements, such as a promoter and enhancer, that allow expression of the DNA segment in the intended target cells of a patient. For expression in blood cells, as is desirable for induction of an immune response, promoter and enhancer elements from light or heavy chain immunoglobulin genes or the CMV major intermediate early promoter and enhancer are suitable to direct expression. The linked regulatory elements and coding sequences are often cloned into a vector. For administration of double-chain antibodies, the two chains can be cloned in the same or separate vectors.

A number of viral vector systems are available including retroviral systems (see, e.g., Lawrie and Tumin, *Cur. Opin. Genet. Develop.* 3, 102-109 (1993)); adenoviral vectors (see, e.g., Bett et al., *J. Virol.* 67, 5911 (1993)); adeno-associated virus vectors (see, e.g., Zhou et al., *J. Exp. Med.* 179, 1867 (1994)), viral vectors from the pox family including vaccinia virus and the avian pox viruses, viral vectors from the alpha virus genus such as those derived from Sindbis and Semliki Forest Viruses (see, e.g., Dubensky et al., *J. Virol.* 70, 508-519 (1996)), and papillomaviruses (Ohe et al., *Human Gene Therapy* 6, 325-333 (1995); Woo et al., WO 94/12629 and Xiao & Brandsma, *Nucleic Acids. Res.* 24, 2630-2622 (1996)).

DNA encoding an immunogen, or a vector containing the same, can be packaged into liposomes. Suitable lipids and related analogs are described by US 5,208,036, 5,264,618, 5,279,833 and 5,283,185. Vectors and DNA encoding an immunogen can also be adsorbed to or associated with particulate carriers, examples of which include polymethyl methacrylate polymers and polylactides and poly(lactide-co-glycolides), see, e.g., McGee et al., *J. Micro Encap.* (1996).

Gene therapy vectors or naked DNA can be delivered in vivo by administration to an individual patient, typically by systemic administration (e.g., intravenous, intraperitoneal, nasal, gastric, intradermal, intramuscular, subdermal, or

intracranial infusion) or topical application (*see e.g.*, US 5,399,346). DNA can also be administered using a gene gun. See Xiao & Brandsma, *supra*. The DNA encoding an immunogen is precipitated onto the surface of microscopic metal beads. The microprojectiles are accelerated with a shock wave or expanding helium gas, and penetrate tissues to a depth of several cell layers. For example, The Accel™ Gene Delivery Device manufactured by Agacetus, Inc. Middleton WI is suitable. Alternatively, naked DNA can pass through skin into the blood stream simply by spotting the DNA onto skin with chemical or mechanical irritation (*see* WO 95/05853).

In a further variation, vectors encoding immunogens can be delivered to cells *ex vivo*, such as cells explanted from an individual patient (e.g., lymphocytes, bone marrow aspirates, tissue biopsy) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient, usually after selection for cells which have incorporated the vector.

III. PATIENTS AMENABLE TO TREATMENT

Patients amenable to treatment include individuals at risk of disease but not showing symptoms, as well as patients presently showing symptoms. In the case of Alzheimer's disease, virtually anyone is at risk of suffering from Alzheimer's disease if he or she lives long enough. Therefore, the present methods can be administered prophylactically to the general population without any assessment of the risk of the subject patient. The present methods are especially useful for individuals who do have a known genetic risk of Alzheimer's disease. Such individuals include those having relatives who have experienced this disease, and those whose risk is determined by analysis of genetic or biochemical markers. Genetic markers of risk toward Alzheimer's disease include mutations in the APP gene, particularly mutations at position 717 and positions 670 and 671 referred to as the Hardy and Swedish mutations respectively (*see* Hardy, TINS, *supra*). Other markers of risk are mutations in the presenilin genes, PS1 and PS2, and ApoE4, family history of AD, hypercholesterolemia or atherosclerosis. Individuals presently suffering from Alzheimer's disease can be recognized from characteristic dementia, as well as the presence of risk factors described above. In addition, a number of diagnostic tests are available for identifying individuals who have AD. These include measurement of CSF tau and A β 42 levels. Elevated tau and decreased A β 42 levels signify the presence of AD. Individuals suffering from

Alzheimer's disease can also be diagnosed by ADRDA criteria as discussed in the Examples section.

In asymptomatic patients, treatment can begin at any age (e.g., 10, 20, 30). Usually, however, it is not necessary to begin treatment until a patient reaches 40, 50, 60 or 70. Treatment typically entails multiple dosages over a period of time. Treatment can be monitored by assaying antibody, or activated T-cell or B-cell responses to the therapeutic agent (e.g., A β peptide) over time. If the response falls, a booster dosage is indicated. In the case of potential Down's syndrome patients, treatment can begin antenatally by administering therapeutic agent to the mother or shortly after birth.

IV. TREATMENT REGIMES

In prophylactic applications, pharmaceutical compositions or medicants are administered to a patient susceptible to, or otherwise at risk of, a particular disease in an amount sufficient to eliminate or reduce the risk or delay the outset of the disease. In therapeutic applications, compositions or medicants are administered to a patient suspected of, or already suffering from such a disease in an amount sufficient to cure, or at least partially arrest, the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as a therapeutically- or pharmaceutically-effective dose. In both prophylactic and therapeutic regimes, agents are usually administered in several dosages until a sufficient immune response has been achieved. Typically, the immune response is monitored and repeated dosages are given if the immune response starts to fade.

Effective doses of the compositions of the present invention, for the treatment of the above described conditions vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic. Usually, the patient is a human, but in some diseases, such as mad cow disease, the patient can be a nonhuman mammal, such as a bovine.

Treatment dosages need to be titrated to optimize safety and efficacy. The amount of immunogen depends on whether adjuvant is also administered, with higher dosages being required in the absence of adjuvant. The amount of an immunogen for administration sometimes varies from 1-500 μ g per patient and more usually from 5-500 μ g per injection for human administration. Occasionally, a higher dose of 1-2 mg per injection is used.

Typically about 10, 20, 50 or 100 μg is used for each human injection. The timing of injections can vary significantly from once a day, to once a year, to once a decade. On any given day that a dosage of immunogen is given, the dosage is greater than 1 μg /patient and usually greater than 10 μg /patient if adjuvant is also administered, and greater than 10 μg /patient and usually greater than 100 μg /patient in the absence of adjuvant. A typical regimen consists of an immunization followed by booster injections at 6 week intervals. Another regimen consists of an immunization followed by booster injections 1, 2 and 12 months later. Another regimen entails an injection every two months for life. Alternatively, booster injections can be on an irregular basis as indicated by monitoring of immune response.

For passive immunization with an antibody, the dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg, of the host body weight. For example dosages can be 1 mg/kg body weight or 10 mg/kg body weight. An exemplary treatment regime entails administration once per every two weeks or once a month or once every 3 to 6 months. In some methods, two or more monoclonal antibodies with different binding specificities are administered simultaneously, in which case the dosage of each antibody administered falls within the ranges indicated. Antibody is usually administered on multiple occasions. Intervals between single dosages can be weekly, monthly or yearly. Intervals can also be irregular as indicated by measuring blood levels of antibody to A β in the patient. Alternatively, antibody can be administered as a sustained release formulation, in which case less frequent administration is required. Dosage and frequency vary depending on the half-life of the antibody in the patient. In general, human antibodies show the longest half life, followed by humanized antibodies, chimeric antibodies, and nonhuman antibodies. The dosage and frequency of administration can vary depending on whether the treatment is prophylactic or therapeutic. In prophylactic applications, a relatively low dosage is administered at relatively infrequent intervals over a long period of time. Some patients continue to receive treatment for the rest of their lives. In therapeutic applications, a relatively high dosage at relatively short intervals is sometimes required until progression of the disease is reduced or terminated, and preferably until the patient shows partial or complete amelioration of symptoms of disease. Thereafter, the patient can be administered a prophylactic regime.

Doses for nucleic acids encoding immunogens range from about 10 ng to 1 g, 100 ng to 100 mg, 1 µg to 10 mg, or 30-300 µg DNA per patient. Doses for infectious viral vectors vary from 10-10⁹, or more, virions per dose.

Agents for inducing an immune response can be administered by
5 parenteral, topical, intravenous, oral, subcutaneous, intraperitoneal, intranasal or intramuscular means for prophylactic and/or therapeutic treatment. The most typical route of administration of an immunogenic agent is subcutaneous although others can be equally effective. The next most common is intramuscular injection. This type of injection is most typically performed in the arm or leg muscles. Intravenous injections as
10 well as intraperitoneal injections, intraarterial, intracranial, or intradermal injections are also effective in generating an immune response. In some methods, agents are injected directly into a particular tissue where deposits have accumulated, for example intracranial injection. Intramuscular injection on intravenous infusion are preferred for administration of antibody. In some methods, particular therapeutic antibodies are injected directly into
15 the cranium. In some methods, antibodies are administered as a sustained release composition or device, such as a MedipadTM device.

Agents of the invention can optionally be administered in combination with other agents that are at least partly effective in treatment of amyloidogenic disease. In the case of Alzheimer's and Down's syndrome, in which amyloid deposits occur in the
20 brain, agents of the invention can also be administered in conjunction with other agents that increase passage of the agents of the invention across the blood-brain barrier.

Immunogenic agents of the invention, such as peptides, are sometimes administered in combination with an adjuvant. A variety of adjuvants can be used in combination with a peptide, such as Aβ, to elicit an immune response. Preferred
25 adjuvants augment the intrinsic response to an immunogen without causing conformational changes in the immunogen that affect the qualitative form of the response. Preferred adjuvants include alum, 3 De-O-acylated monophosphoryl lipid A (MPL) (see GB 2220211). QS-21 is a triterpene glycoside or saponin isolated from the bark of the Quillaja Saponaria Molina tree found in South America (see Kensil et al., in *Vaccine*
30 *Design: The Subunit and Adjuvant Approach* (eds. Powell & Newman, Plenum Press, NY, 1995); US Patent No. 5,057,540). Other adjuvants are oil in water emulsions (such as squalene or peanut oil), optionally in combination with immune stimulants, such as monophosphoryl lipid A (see Stoute et al., *N. Engl. J. Med.* 336, 86-91 (1997)). Another

adjuvant is CpG (*Bioworld Today*, Nov. 15, 1998). Alternatively, A β can be coupled to an adjuvant. However, such coupling should not substantially change the conformation of A β so as to affect the nature of the immune response thereto. Adjuvants can be administered as a component of a therapeutic composition with an active agent or can be administered separately, before, concurrently with, or after administration of the therapeutic agent.

A preferred class of adjuvants is aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate. Such adjuvants can be used with or without other specific immunostimulating agents such as MPL or 3-DMP, QS-21, polymeric or monomeric amino acids such as polyglutamic acid or polylysine. Another class of adjuvants is oil-in-water emulsion formulations. Such adjuvants can be used with or without other specific immunostimulating agents such as muramyl peptides (*e.g.*, N-acetylmuramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), N-acetylglucosaminyl-N-acetylmuramyl-L-Al-D-isoglu-L-Ala-dipalmitoxy propylamide (DTP-DPP) theramideTM), or other bacterial cell wall components. Oil-in-water emulsions include (a) MF59 (WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton MA), (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) RibiTM adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (DetoxTM). Another class of preferred adjuvants is saponin adjuvants, such as StimulonTM (QS-21, Aquila, Worcester, MA) or particles generated therefrom such as ISCOMs (immunostimulating complexes) and ISCOMATRIX. Other adjuvants include Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA). Other adjuvants include cytokines, such as interleukins (IL-1, IL-2, and IL-12), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF).

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An adjuvant can be administered with an immunogen as a single composition, or can be administered before, concurrent with or after administration of the immunogen. Immunogen and adjuvant can be packaged and supplied in the same vial or can be packaged in separate vials and mixed before use. Immunogen and adjuvant are typically packaged with a label indicating the intended therapeutic application. If immunogen and adjuvant are packaged separately, the packaging typically includes instructions for mixing before use. The choice of an adjuvant and/or carrier depends on the stability of the vaccine containing the adjuvant, the route of administration, the dosing schedule, the efficacy of the adjuvant for the species being vaccinated, and, in humans, a pharmaceutically acceptable adjuvant is one that has been approved or is approvable for human administration by pertinent regulatory bodies. For example, Complete Freund's adjuvant is not suitable for human administration. Alum, MPL and QS-21 are preferred. Optionally, two or more different adjuvants can be used simultaneously. Preferred combinations include alum with MPL, alum with QS-21, MPL with QS-21, and alum, QS-21 and MPL together. Also, Incomplete Freund's adjuvant can be used (Chang *et al.*, *Advanced Drug Delivery Reviews* 32, 173-186 (1998)), optionally in combination with any of alum, QS-21, and MPL and all combinations thereof.

Agents of the invention are often administered as pharmaceutical compositions comprising an active therapeutic agent, i.e., and a variety of other pharmaceutically acceptable components. See *Remington's Pharmaceutical Science* (15th ed., Mack Publishing Company, Easton, Pennsylvania, 1980). The preferred form depends on the intended mode of administration and therapeutic application. The compositions can also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers or diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, physiological phosphate-buffered saline, Ringer's solutions, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation may also include other carriers, adjuvants, or nontoxic, nontherapeutic, nonimmunogenic stabilizers and the like. However, some reagents suitable for administration to animals, such as Complete Freund's adjuvant are not typically included in compositions for human use.

Pharmaceutical compositions can also include large, slowly metabolized macromolecules such as proteins, polysaccharides such as chitosan, polylactic acids,

polyglycolic acids and copolymers (such as latex functionalized sepharose, agarose, cellulose, and the like), polymeric amino acids, amino acid copolymers, and lipid aggregates (such as oil droplets or liposomes). Additionally, these carriers can function as immunostimulating agents (i.e., adjuvants).

5 For parenteral administration, agents of the invention can be administered as injectable dosages of a solution or suspension of the substance in a physiologically acceptable diluent with a pharmaceutical carrier that can be a sterile liquid such as water oils, saline, glycerol, or ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents, surfactants, pH buffering substances and the like can be present in
10 compositions. Other components of pharmaceutical compositions are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, and mineral oil. In general, glycols such as propylene glycol or polyethylene glycol are preferred liquid carriers, particularly for injectable solutions. Antibodies can be administered in the form of a depot injection or implant preparation which can be formulated in such a
15 manner as to permit a sustained release of the active ingredient. An exemplary composition comprises monoclonal antibody at 5 mg/mL, formulated in aqueous buffer consisting of 50 mM L-histidine, 150 mM NaCl, adjusted to pH 6.0 with HCl.

Typically, compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid
20 vehicles prior to injection can also be prepared. The preparation also can be emulsified or encapsulated in liposomes or micro particles such as polylactide, polyglycolide, or copolymer for enhanced adjuvant effect, as discussed above (see Langer, *Science* 249, 1527 (1990) and Hanes, *Advanced Drug Delivery Reviews* 28, 97-119 (1997)). The agents of this invention can be administered in the form of a depot injection or implant
25 preparation which can be formulated in such a manner as to permit a sustained or pulsatile release of the active ingredient.

Additional formulations suitable for other modes of administration include oral, intranasal, and pulmonary formulations, suppositories, and transdermal applications.

For suppositories, binders and carriers include, for example, polyalkylene glycols or triglycerides; such suppositories can be formed from mixtures containing the
30 active ingredient in the range of 0.5% to 10%, preferably 1%-2%. Oral formulations include excipients, such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, and magnesium carbonate. These compositions

take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25%-70%.

Topical application can result in transdermal or intradermal delivery.

Topical administration can be facilitated by co-administration of the agent with cholera toxin or detoxified derivatives or subunits thereof or other similar bacterial toxins (See Glenn *et al.*, *Nature* 391, 851 (1998)). Co-administration can be achieved by using the components as a mixture or as linked molecules obtained by chemical crosslinking or expression as a fusion protein.

Alternatively, transdermal delivery can be achieved using a skin patch or using transferosomes (Paul *et al.*, *Eur. J. Immunol.* 25, 3521-24 (1995); Cevc *et al.*, *Biochem. Biophys. Acta* 1368, 201-15 (1998)).

V. Methods of Diagnosis

The invention provides methods of detecting an immune response against A β peptide in a patient suffering from or susceptible to Alzheimer's disease. The methods are particularly useful for monitoring a course of treatment being administered to a patient. The methods can be used to monitor both therapeutic treatment on symptomatic patients and prophylactic treatment on asymptomatic patients. The methods are useful for monitoring both active immunization (e.g., antibody produced in response to administration of immunogen) and passive immunization (e.g., measuring level of administered antibody).

1. Active Immunization

Some methods entail determining a baseline value of an immune response in a patient before administering a dosage of agent, and comparing this with a value for the immune response after treatment. A significant increase (i.e., greater than the typical margin of experimental error in repeat measurements of the same sample, expressed as one standard deviation from the mean of such measurements) in value of the immune response signals a positive treatment outcome (i.e., that administration of the agent has achieved or augmented an immune response). If the value for immune response does not change significantly, or decreases, a negative treatment outcome is indicated. In general, patients undergoing an initial course of treatment with an immunogenic agent are expected to show an increase in immune response with successive dosages, which

eventually reaches a plateau. Administration of agent is generally continued while the immune response is increasing. Attainment of the plateau is an indicator that the administered of treatment can be discontinued or reduced in dosage or frequency.

5 In other methods, a control value (i.e., a mean and standard deviation) of immune response is determined for a control population. Typically the individuals in the control population have not received prior treatment. Measured values of immune response in a patient after administering a therapeutic agent are then compared with the control value. A significant increase relative to the control value (e.g., greater than one standard deviation from the mean) signals a positive treatment outcome. A lack of
10 significant increase or a decrease signals a negative treatment outcome. Administration of agent is generally continued while the immune response is increasing relative to the control value. As before, attainment of a plateau relative to control values is an indicator that the administration of treatment can be discontinued or reduced in dosage or frequency.

15 In other methods, a control value of immune response (e.g., a mean and standard deviation) is determined from a control population of individuals who have undergone treatment with a therapeutic agent and whose immune responses have plateaued in response to treatment. Measured values of immune response in a patient are compared with the control value. If the measured level in a patient is not significantly
20 different (e.g., more than one standard deviation) from the control value, treatment can be discontinued. If the level in a patient is significantly below the control value, continued administration of agent is warranted. If the level in the patient persists below the control value, then a change in treatment regime, for example, use of a different adjuvant may be indicated.

25 In other methods, a patient who is not presently receiving treatment but has undergone a previous course of treatment is monitored for immune response to determine whether a resumption of treatment is required. The measured value of immune response in the patient can be compared with a value of immune response previously achieved in the patient after a previous course of treatment. A significant decrease
30 relative to the previous measurement (i.e., greater than a typical margin of error in repeat measurements of the same sample) is an indication that treatment can be resumed. Alternatively, the value measured in patient can be compared with a control value (mean plus standard deviation) determined in population of patients after undergoing a course of treatment. Alternatively, the measured value in a patient can be compared with a control

value in populations of prophylactically treated patients who remain free of symptoms of disease, or populations of therapeutically treated patients who show amelioration of disease characteristics. In all of these cases, a significant decrease relative to the control level (i.e., more than a standard deviation) is an indicator that treatment should be resumed in a patient.

The tissue sample for analysis is typically blood, plasma, serum, mucous or cerebrospinal fluid from the patient. The sample is analyzed for indication of an immune response to any form of A β peptide, typically A β 42. The immune response can be determined from the presence of, e.g., antibodies or T-cells that specifically bind to A β peptide. ELISA methods of detecting antibodies specific to A β are described in the Examples section. Methods of detecting reactive T-cells have been described above (see Definitions).

2. Passive Immunization

In general, the procedures for monitoring passive immunization are similar to those for monitoring active immunization described above. However, the antibody profile following passive immunization typically shows an immediate peak in antibody concentration followed by an exponential decay. Without a further dosage, the decay approaches pretreatment levels within a period of days to months depending on the half-life of the antibody administered. For example the half-life of some human antibodies is of the order of 20 days.

In some methods, a baseline measurement of antibody to A β in the patient is made before administration, a second measurement is made soon thereafter to determine the peak antibody level, and one or more further measurements are made at intervals to monitor decay of antibody levels. When the level of antibody has declined to baseline or a predetermined percentage of the peak less baseline (e.g., 50%, 25% or 10%), administration of a further dosage of antibody is administered. In some methods, peak or subsequent measured levels less background are compared with reference levels previously determined to constitute a beneficial prophylactic or therapeutic treatment regime in other patients. If the measured antibody level is significantly less than a reference level (e.g., less than the mean minus one standard deviation of the reference value in population of patients benefiting from treatment) administration of an additional dosage of antibody is indicated.

3. Diagnostic Kits

The invention further provides diagnostic kits for performing the diagnostic methods described above. Typically, such kits contain an agent that specifically binds to antibodies to A β or reacts with T-cells specific for A β . The kit can also include a label. For detection of antibodies to A β , the label is typically in the form of labelled anti-idiotypic antibodies. For detection of antibodies, the agent can be supplied prebound to a solid phase, such as to the wells of a microtiter dish. For detection of reactive T-cells, the label can be supplied as 3H-thymidine to measure a proliferative response. Kits also typically contain labelling providing directions for use of the kit. The labelling may also include a chart or other correspondence regime correlating levels of measured label with levels of antibodies to A β or T-cells reactive with A β . The term labelling refers to any written or recorded material that is attached to, or otherwise accompanies a kit at any time during its manufacture, transport, sale or use. For example, the term labelling encompasses advertising leaflets and brochures, packaging materials, instructions, audio or video cassettes, computer discs, as well as writing imprinted directly on kits.

EXAMPLES

I. PROPHYLACTIC EFFICACY OF A β AGAINST AD

These examples describe administration of A β ₄₂ peptide to transgenic mice overexpressing APP with a mutation at position 717 (APP_{717V→F}) that predisposes them to develop Alzheimer's-like neuropathology. Production and characteristics of these mice (PDAPP mice) is described in Games et al., *Nature, supra*. These animals, in their heterozygote form, begin to deposit A β at six months of age forward. By fifteen months of age they exhibit levels of A β deposition equivalent to that seen in Alzheimer's disease. PDAPP mice were injected with aggregated A β ₄₂ (aggregated A β ₄₂) or phosphate buffered saline. Aggregated A β ₄₂ was chosen because of its ability to induce antibodies to multiple epitopes of A β .

A. METHODS

1. Source of Mice

Thirty PDAPP heterogenic female mice were randomly divided into the following groups: 10 mice to be injected with aggregated A β 42 (one died in transit), 5 mice to be injected with PBS/adjuvant or PBS, and 10 uninjected controls. Five mice were injected with peptides derived from the sequence of serum amyloid protein (SAP).

2. Preparation of Immunogens

Preparation of aggregated A β 42: two milligrams of A β 42 (US Peptides Inc, lot K-42-12) was dissolved in 0.9 ml water and made up to 1 ml by adding 0.1 ml 10 x PBS. This was vortexed and allowed to incubate overnight 37° C, under which conditions the peptide aggregated. Any unused A β was stored as a dry lyophilized powder at -20° C until the next injection.

3. Preparation of Injections

For each injection, 100 μ g of aggregated A β 42 in PBS per mouse was emulsified 1:1 with Complete Freund's adjuvant (CFA) in a final volume of 400 μ l emulsion for the first immunization, followed by a boost of the same amount of immunogen in Incomplete Freund's adjuvant (IFA) at 2 weeks. Two additional doses in IFA were given at monthly intervals. The subsequent immunizations were done at monthly intervals in 500 μ l of PBS. Injections were delivered intraperitoneally (i.p.).

PBS injections followed the same schedule and mice were injected with a 1:1 mix of PBS/ Adjuvant at 400 μ l per mouse, or 500 μ l of PBS per mouse. SAP injections likewise followed the same schedule using a dose of 100 μ g per injection.

4. Titration of Mouse Bleeds, Tissue Preparation and Immunohistochemistry

The above methods are described infra in General Materials and Methods.

B. RESULTS

PDAPP mice were injected with either aggregated A β 42 (aggregated A β 42), SAP peptides, or phosphate buffered saline. A group of PDAPP mice were also left as uninjected, positive controls. The titers of the mice to aggregated A β 42 were

monitored every other month from the fourth boost until the mice were one year of age. Mice were sacrificed at 13 months. At all time points examined, eight of the nine aggregated A β 42 mice developed a high antibody titer, which remained high throughout the series of injections (titers greater than 1/10000). The ninth mouse had a low, but measurable titer of approximately 1/1000 (Figure 1, Table 1). SAPP-injected mice had titers of 1:1,000 to 1:30,000 for this immunogen with only a single mouse exceeding 1:10,000.

The PBS-treated mice were titrated against aggregated A β 42 at six, ten and twelve months. At a 1/100 dilution the PBS mice when titrated against aggregated A β 42 only exceeded 4 times background at one data point, otherwise, they were less than 4 times background at all time points (Table 1). The SAP-specific response was negligible at these time points with all titers less than 300.

Seven out of the nine mice in the aggregated A β 1-42 treated group had no detectable amyloid in their brains. In contrast, brain tissue from mice in the SAP and PBS groups contained numerous amyloid deposits in the hippocampus, as well as in the frontal and cingulate cortices. The pattern of deposition was similar to that of untreated controls, with characteristic involvement of vulnerable subregions, such as the outer molecular layer of the hippocampal dentate gyrus. One mouse from the A β 1-42-injected group had a greatly reduced amyloid burden, confined to the hippocampus. An isolated plaque was identified in another A β 1-42-treated mouse.

Quantitative image analyses of the amyloid burden in the hippocampus verified the dramatic reduction achieved in the A β 42(AN1792) -treated animals (Fig. 2). The median values of the amyloid burden for the PBS group (2.22%), and for the untreated control group (2.65%) were significantly greater than for those immunized with AN1792 (0.00%, $p=0.0005$). In contrast, the median value for the group immunized with SAP peptides (SAPP) was 5.74%. Brain tissue from the untreated, control mice contained numerous A β amyloid deposits visualized with the A β -specific monoclonal antibody (mAb) 3D6 in the hippocampus, as well as in the retrosplenial cortex. A similar pattern of amyloid deposition was also seen in mice immunized with SAPP or PBS (Fig. 2). In addition, in these latter three groups there was a characteristic involvement of vulnerable subregions of the brain classically seen in AD, such as the outer molecular layer of the hippocampal dentate gyrus, in all three of these groups.

The brains that contained no A β deposits were also devoid of neuritic plaques that are typically visualized in PDAPP mice with the human APP antibody 8E5. All of brains from the remaining groups (SAP-injected, PBS and uninjected mice) had numerous neuritic plaques typical of untreated PDAPP mice. A small number of neuritic
5 plaques were present in one mouse treated with AN1792, and a single cluster of dystrophic neurites was found in a second mouse treated with AN1792. Image analyses of the hippocampus, and shown in Fig. 3, demonstrated the virtual elimination of dystrophic neurites in AN1792-treated mice (median 0.00%) compared to the PBS recipients (median 0.28%, $p = 0.0005$).

10 Astrocytosis characteristic of plaque-associated inflammation was also absent in the brains of the A β 1-42 injected group. The brains from the mice in the other groups contained abundant and clustered GFAP-positive astrocytes typical of A β plaque-associated gliosis. A subset of the GFAP-reacted slides were counter-stained with Thioflavin S to localize the A β deposits. The GFAP-positive astrocytes were associated
15 with A β plaques in the SAP, PBS and untreated controls. No such association was found in the plaque-negative A β 1-42 treated mice, while minimal plaque-associated gliosis was identified in one mouse treated with AN1792.

Image analyses, shown in Fig. 4 for the retrosplenial cortex, verified that the reduction in astrocytosis was significant with a median value of 1.56% for those
20 treated with AN1792 versus median values greater than 6% for groups immunized with SAP peptides, PBS or untreated ($p=0.0017$)

Evidence from a subset of the A β 1-42- and PBS-injected mice indicated plaque-associated MHC II immunoreactivity was absent in the A β 1-42 injected mice, consistent with lack of an A β -related inflammatory response.

25 Sections of the mouse brains were also reacted with a mAb specific with a monoclonal antibody specific for MAC-1, a cell surface protein. MAC-1 (CD11b) is an integrin family member and exists as a heterodimer with CD18. The CD11b/CD18 complex is present on monocytes, macrophages, neutrophils and natural killer cells (Mak and Simard). The resident MAC-1-reactive cell type in the brain is likely to be microglia
30 based on similar phenotypic morphology in MAC-1 immunoreacted sections. Plaque-associated MAC-1 labeling was lower in the brains of mice treated with AN1792 compared to the PBS control group, a finding consistent with the lack of an A β -induced inflammatory response.

C. CONCLUSION

The lack of A β plaques and reactive neuronal and gliotic changes in the brains of the A β 1-42-injected mice indicate that no or extremely little amyloid was deposited in their brains, and pathological consequences, such as gliosis and neuritic pathology, were absent. PDAPP mice treated with A β 1-42 show essentially the same lack of pathology as control nontransgenic mice. Therefore, A β 1-42 injections are highly effective in the prevention of deposition or clearance of human A β from brain tissue, and elimination of subsequent neuronal and inflammatory degenerative changes. Thus, administration of A β peptide can have both preventative and therapeutic benefit in prevention of AD.

II. DOSE RESPONSE STUDY

Groups of five-week old, female Swiss Webster mice (N=6 per group) were immunized with 300, 100, 33, 11, 3.7, 1.2, 0.4, or 0.13 μ g of A β formulated in CFA/IFA administered intraperitoneally. Three doses were given at biweekly intervals followed by a fourth dose one month later. The first dose was emulsified with CFA and the remaining doses were emulsified with IFA. Animals were bled 4-7 days following each immunization starting after the second dose for measurement of antibody titers. Animals in a subset of three groups, those immunized with 11, 33, or 300 μ g of antigen, were additionally bled at approximately monthly intervals for four months following the fourth immunization to monitor the decay of the antibody response across a range of vaccine doses. These animals received a final fifth immunization at seven months after study initiation. They were sacrificed one week later to measure antibody responses to AN1792 and to perform toxicological analyses.

A declining dose response was observed from 300 to 3.7 μ g with no response at the two lowest doses. Mean antibody titers are about 1:1000 after 3 doses and about 1:10,000 after 4 doses of 11-300 μ g of antigen (see Fig. 5).

Antibody titers rose dramatically for all but the lowest dose group following the third immunization with increases in GMTs ranging from 5- to 25-fold. Low antibody responses were then detectable for even the 0.4 μ g recipients. The 1.2 and 3.7 μ g groups had comparable titers with GMTs of about 1000 and the highest four doses clustered together with GMTs of about 25,000, with the exception of the 33 μ g dose

group with a lower GMT of 3000. Following the fourth immunization, the titer increase was more modest for most groups. There was a clear dose response across the lower antigen dose groups from 0.14 μ g to 11 μ g ranging from no detectable antibody for recipients of 0.14 μ g to a GMT of 36,000 for recipients of 11 μ g. Again, titers for the four highest dose groups of 11 to 300 μ g clustered together. Thus following two immunizations, the antibody titer was dependent on the antigen dose across the broad range from 0.4 to 300 μ g. By the third immunization, titers of the highest four doses were all comparable and they remained at a plateau after an additional immunization.

One month following the fourth immunization, titers were 2- to 3-fold higher in the 300 μ g group than those measured from blood drawn five days following the immunization (Fig. 6). This observation suggests that the peak anamnestic antibody response occurred later than 5 days post-immunization. A more modest (50%) increase was seen at this time in the 33 μ g group. In the 300 μ g dose group at two months following the last dose, GMTs declined steeply by about 70%. After another month, the decline was less steep at 45% (100 μ g) and about 14% for the 33 and 11 μ g doses. Thus, the rate of decline in circulating antibody titers following cessation of immunization appears to be biphasic with a steep decline the first month following peak response followed by a more modest rate of decrease thereafter.

The antibody titers and the kinetics of the response of these Swiss Webster mice are similar to those of young heterozygous PDAPP transgenic mice immunized in a parallel manner. Dosages effective to induce an immune response in humans are typically similar to dosages effective in mice.

III. SCREEN FOR THERAPEUTIC EFFICACY AGAINST ESTABLISHED AD

This assay is designed to test immunogenic agents for activity in arresting or reversing neuropathologic characteristics of AD in aged animals. Immunizations with 42 amino acid long A β (AN1792) were begun at a time point when amyloid plaques are already present in the brains of the PDAPP mice.

Over the time course used in this study, untreated PDAPP mice develop a number of neurodegenerative changes that resemble those found in AD (*Games et al., supra* and Johnson-Wood et al., *Proc. Natl. Acad. Sci. USA* 94, 1550-1555 (1997)). The deposition of A β into amyloid plaques is associated with a degenerative neuronal response consisting of aberrant axonal and dendritic elements, called dystrophic neurites.

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Amyloid deposits that are surrounded by and contain dystrophic neurites called neuritic plaques. In both AD and the PDAPP mouse, dystrophic neurites have a distinctive globular structure, are immunoreactive with a panel of antibodies recognizing APP and cytoskeletal components, and display complex subcellular degenerative changes at the ultrastructural level. These characteristics allow for disease-relevant, selective and reproducible measurements of neuritic plaque formation in the PDAPP brains. The dystrophic neuronal component of PDAPP neuritic plaques is easily visualized with an antibody specific for human APP (monoclonal antibody 8E5), and is readily measurable by computer-assisted image analysis. Therefore, in addition to measuring the effects of AN1792 on amyloid plaque formation, we monitored the effects of this treatment on the development of neuritic dystrophy.

Astrocytes and microglia are non-neuronal cells that respond to and reflect the degree of neuronal injury. GFAP-positive astrocytes and MHC II-positive microglia are commonly observed in AD, and their activation increases with the severity of the disease. Therefore, we also monitored the development of reactive astrocytosis and microgliosis in the AN1792-treated mice.

A. Materials and Methods

Forty-eight, heterozygous female PDAPP mice, 11 to 11.5 months of age, obtained from Charles River, were randomly divided into two groups: 24 mice to be immunized with 100 μ g of AN1792 and 24 mice to be immunized with PBS, each combined with Freund's adjuvant. The AN1792 and PBS groups were again divided when they reached ~15 months of age. At 15 months of age approximately half of each group of the AN1792- and PBS-treated animals were euthanized (n=10 and 9, respectively), the remainder continued to receive immunizations until termination at ~18 months (n=9 and 12, respectively). A total of 8 animals (5 AN1792, 3 PBS) died during the study. In addition to the immunized animals, one-year old (n=10), 15-month old (n=10) and 18-month old (n=10) untreated PDAPP mice were included for comparison in the ELISAs to measure $a\beta$ and APP levels in the brain; the one-year old animals were also included in the immunohistochemical analyses.

Methodology was as in Example 1 unless otherwise indicated. US Peptides lot 12 and California Peptides lot ME0339 of AN1792 were used to prepare the antigen for the six immunizations administered prior to the 15-month time point.

California Peptides lots ME0339 and ME0439 were used for the three additional immunizations administered between 15 and 18 months.

For immunizations, 100 µg of AN1792 in 200 µl PBS or PBS alone was emulsified 1:1 (vol:vol) with Complete Freund's adjuvant (CFA) or Incomplete Freund's adjuvant (IFA) or PBS in a final volume of 400 µl. The first immunization was delivered with CFA as adjuvant, the next four doses were given with IFA and the final four doses with PBS alone without added adjuvant. A total of nine immunizations were given over the seven-month period on a two-week schedule for the first three doses followed by a four-week interval for the remaining injections. The four-month treatment group, euthanized at 15 months of age, received only the first 6 immunizations.

B. Results

1. Effects of AN1792 Treatment on Amyloid Burden

The results of AN1792 treatment on cortical amyloid burden determined by quantitative image analysis are shown in Fig. 7. The median value of cortical amyloid burden was 0.28% in a group of untreated 12-month old PDAPP mice, a value representative of the plaque load in mice at the study's initiation. At 18 months, the amyloid burden increased over 17-fold to 4.87% in PBS-treated mice, while AN1792-treated mice had a greatly reduced amyloid burden of only 0.01%, notably less than the 12-month untreated and both the 15- and 18-month PBS-treated groups. The amyloid burden was significantly reduced in the AN1792 recipients at both 15 (96% reduction; $p=0.003$) and 18 (>99% reduction; $p=0.0002$) months.

Typically, cortical amyloid deposition in PDAPP mice initiates in the frontal and retrosplenial cortices (RSC) and progresses in a ventral-lateral direction to involve the temporal and entorhinal cortices (EC). Little or no amyloid was found in the EC of 12 month-old mice, the approximate age at which AN1792 was first administered. After 4 months of AN1792 treatment, amyloid deposition was greatly diminished in the RSC, and the progressive involvement of the EC was entirely eliminated by AN1792 treatment. The latter observation showed that AN1792 completely halted the progression of amyloid that would normally invade the temporal and ventral cortices, as well as arrested or possibly reversed deposition in the RSC.

The profound effects of AN1792 treatment on developing cortical amyloid burden in the PDAPP mice are further demonstrated by the 18-month group, which had

been treated for seven months. A near complete absence of cortical amyloid was found in the AN1792-treated mouse, with a total lack of diffuse plaques, as well as a reduction in compacted deposits.

5 2. AN1792 Treatment-associated Cellular and Morphological Changes

 A population of A β -positive cells was found in brain regions that typically contain amyloid deposits. Remarkably, in several brains from AN1792 recipients, very few or no extracellular cortical amyloid plaques were found. Most of the A β
10 immunoreactivity appeared to be contained within cells with large lobular or clumped soma. Phenotypically, these cells resembled activated microglia or monocytes. They were immunoreactive with antibodies recognizing ligands expressed by activated monocytes and microglia (MHC II and CD11b) and were occasionally associated with the wall or lumen of blood vessels. Comparison of near-adjacent sections labeled with A β
15 and MHC II-specific antibodies revealed that similar patterns of these cells were recognized by both classes of antibodies. Detailed examination of the AN1792-treated brains revealed that the MHC II-positive cells were restricted to the vicinity of the limited amyloid remaining in these animals. Under the fixation conditions employed, the cells were not immunoreactive with antibodies that recognize T cell (CD3, CD3e) or B cell
20 (CD45RA, CD45RB) ligands or leukocyte common antigen (CD45), but were reactive with an antibody recognizing leukosialin (CD43) which cross-reacts with monocytes. No such cells were found in any of the PBS-treated mice.

 PDAPP mice invariably develop heavy amyloid deposition in the outer molecular layer of the hippocampal dentate gyrus. The deposition forms a distinct streak
25 within the perforant pathway, a subregion that classically contains amyloid plaques in AD. The characteristic appearance of these deposits in PBS-treated mice resembled that previously characterized in untreated PDAPP mice. The amyloid deposition consisted of both diffuse and compacted plaques in a continuous band. In contrast, in a number of brains from AN1792-treated mice this pattern was drastically altered. The hippocampal
30 amyloid deposition no longer contained diffuse amyloid, and the banded pattern was completely disrupted. Instead, a number of unusual punctate structures were present that are reactive with anti-A β antibodies, several of which appeared to be amyloid-containing cells.

MHC II-positive cells were frequently observed in the vicinity of extracellular amyloid in AN1792-treated animals. The pattern of association of A β -positive cells with amyloid was very similar in several brains from AN1792-treated mice. The distribution of these monocytic cells was restricted to the proximity of the deposited amyloid and was entirely absent from other brain regions devoid of A β plaques.

Quantitative image analysis of MHC II and MAC I-labeled sections revealed a trend towards increased immunoreactivity in the RSC and hippocampus of AN1792-treated mice compared to the PBS group which reached significance with the measure of MAC 1 reactivity in hippocampus.

These results are indicative of active, cell-mediated removal of amyloid in plaque-bearing brain regions.

3. AN1792 Effects on A β Levels: ELISA Determinations

(a) Cortical Levels

In untreated PDAPP mice, the median level of total A β in the cortex at 12 months was 1,600 ng/g, which increased to 8,700 ng/g by 15 months (Table 2). At 18 months the value was 22,000 ng/g, an increase of over 10-fold during the time course of the experiment. PBS-treated animals had 8,600 ng/g total A β at 15 months which increased to 19,000 ng/g at 18 months. In contrast, AN1792-treated animals had 81% less total A β at 15 months (1,600 ng/g) than the PBS-immunized group. Significantly less ($p=0.0001$) total A β (5,200 ng/g) was found at 18 months when the AN1792 and PBS groups were compared (Table 2), representing a 72% reduction in the A β that would otherwise be present. Similar results were obtained when cortical levels of A β 42 were compared, namely that the AN1792-treated group contained much less A β 42, but in this case the differences between the AN1792 and PBS groups were significant at both 15 months ($p=0.04$) and 18 months ($p=0.0001$, Table 2).

Table 2: Median A β Levels (ng/g) in Cortex

Age	UNTREATED			PBS			AN1792		
	Total	A β 42	(n)	T tal	A β 42	(n)	Total	A β 42	(n)
12	1,600	1,300	(10)						
15	8,700	8,300	(10)	8,600	7,200	(9)	1,600	1,300*	(10)
18	22,200	18,500	(10)	19,000	15,900	(12)	5,200**	4,000**	(9)

* p = 0.0412

** p = 0.0001

(b) Hippocampal Levels

In untreated PDAPP mice, median hippocampal levels of total A β at twelve months of age were 15,000 ng/g which increased to 51,000 ng/g at 15 months and further to 81,000 ng/g at 18 months (Table 3). Similarly, PBS immunized mice showed values of 40,000 ng/g and 65,000 ng/g at 15 months and 18 months, respectively. AN1792 immunized animals exhibited less total A β , specifically 25,000 ng/g and 51,000 ng/g at the respective 15-month and 18-month timepoints. The 18-month AN1792-treated group value was significantly lower than that of the PBS treated group (p= 0.0105; Table 3). Measurement of A β 42 gave the same pattern of results, namely that levels in the AN1792-treated group were significantly lower than in the PBS group (39,000 ng/g vs. 57,000 ng/g, respectively; p=0.002) at the 18-month evaluation (Table 3).

Table 3: Median A β Levels (ng/g) in Hippocampus

Age	UNTREATED			PBS			AN1792		
	Total	A β 42	(n)	Total	A β 42	(n)	Total	A β 42	(n)
12	15,500	11,100	(10)						
15	51,500	44,400	(10)	40,100	35,70	(9)	24,50	22,100	(10)
18	80,800	64,200	(10)	65,400	57,10	(12)	50,90	38,900**	(9)

* p = 0.0105

** p = 0.0022

(c) Cerebellar Levels

In 12-month untreated PDAPP mice, the median cerebellar level of total A β was 15 ng/g (Table 4). At 15 months, this median increased to 28 ng/g and by 18 months had risen to 35 ng/g. PBS-treated animals displayed median total A β values of 21 ng/g at 15 months and 43 ng/g at 18 months. AN1792-treated animals were found to have 22 ng/g total A β at 15 months and significantly less (p=0.002) total A β at 18 months (25 ng/g) than the corresponding PBS group (Table 4).

Table 4: Median A β Levels (ng/g) in Cerebellum

Age	UNTREATED		PBS		AN1792	
	Total A β	(n)	Total A β	(n)	Total A β	(n)
12	15.6	(10)				
15	27.7	(10)	20.8	(9)	21.7	(10)
18	35.0	(10)	43.1	(12)	24.8*	(9)

* p = 0.0018

4. Effects of AN1792 Treatment on APP Levels

APP- α and the full-length APP molecule both contain all or part of the A β sequence and thus could be potentially impacted by the generation of an AN1792-directed immune response. In studies to date, a slight increase in APP levels has been noted as neuropathology increases in the PDAPP mouse. In the cortex, levels of either APP- α /FL (full length) or APP- α were essentially unchanged by treatment with the exception that APP- α was reduced by 19% at the 18-month timepoint in the AN1792-treated vs. the PBS-treated group. The 18-month AN1792-treated APP values were not significantly different from values of the 12-month and 15-month untreated and 15-month PBS groups. In all cases the APP values remained within the ranges that are normally found in PDAPP mice.

5. Effects of AN1792 Treatment on Neurodegenerative and Gliotic Pathology

Neuritic plaque burden was significantly reduced in the frontal cortex of AN1792-treated mice compared to the PBS group at both 15 (84%; p=0.03) and 18 (55%; p=0.01) months of age (Fig. 8). The median value of the neuritic plaque burden increased from 0.32% to 0.49% in the PBS group between 15 and 18 months of age. This contrasted with the greatly reduced development of neuritic plaques in the AN1792 group, with median neuritic plaque burden values of 0.05% and 0.22%, in the 15 and 18 month groups, respectively.

Immunizations with AN1792 seemed well tolerated and reactive astrocytosis was also significantly reduced in the RSC of AN1792-treated mice when compared to the PBS group at both 15 (56%; p=0.011) and 18 (39%; p=0.028) months of age (Fig. 9). Median values of the percent of astrocytosis in the PBS group increased between 15 and 18 months from 4.26% to 5.21%. AN1792-treatment suppressed the

development of astrocytosis at both time points to 1.89% and 3.2%, respectively. This suggests the neuropil was not being damaged by the clearance process.

6. Antibody Responses

As described above, eleven-month old, heterozygous PDAPP mice (N=24) received a series of 5 immunizations of 100 μ g of AN1792 emulsified with Freund's adjuvant and administered intraperitoneally at weeks 0, 2, 4, 8, and 12, and a sixth immunization with PBS alone (no Freund's adjuvant) at week 16. As a negative control, a parallel set of 24 age-matched transgenic mice received immunizations of PBS emulsified with the same adjuvants and delivered on the same schedule. Animals were bled within three to seven days following each immunization starting after the second dose. Antibody responses to AN1792 were measured by ELISA. Geometric mean titers (GMT) for the animals that were immunized with AN1792 were approximately 1,900, 7,600, and 45,000 following the second, third and last (sixth) doses respectively. No A β -specific antibody was measured in control animals following the sixth immunization.

Approximately one-half of the animals were treated for an additional three months, receiving immunizations at about 20, 24 and 27 weeks. Each of these doses was delivered in PBS vehicle alone without Freund's adjuvant. Mean antibody titers remained unchanged over this time period. In fact, antibody titers appeared to remain stable from the fourth to the eighth bleed corresponding to a period covering the fifth to the ninth injections.

To determine if the A β -specific antibodies elicited by immunization that were detected in the sera of AN1792-treated mice were also associated with deposited brain amyloid, a subset of sections from the AN1792- and PBS-treated mice were reacted with an antibody specific for mouse IgG. In contrast to the PBS group, A β plaques in AN1792-treated brains were coated with endogenous IgG. This difference between the two groups was seen in both 15- and 18-month groups. Particularly striking was the lack of labeling in the PBS group, despite the presence of a heavy amyloid burden in these mice. These results show that immunization with a synthetic A β protein generates antibodies that recognize and bind in vivo to the A β in amyloid plaques.

7. Cellular-Mediated Immune Responses

Spleens were removed from nine AN1792-immunized and 12 PBS-immunized 18-month old PDAPP mice 7 days after the ninth immunization. Splenocytes were isolated and cultured for 72 h in the presence of A β 40, A β 42, or A β 40-1 (reverse order protein). The mitogen Con A served as a positive control. Optimum responses were obtained with >1.7 μ M protein. Cells from all nine AN1792-treated animals proliferated in response to either A β 1-40 or A β 1-42 protein, with equal levels of incorporation for both proteins (Fig. 10, Upper Panel). There was no response to the A β 40-1 reverse protein. Cells from control animals did not respond to any of the A β proteins (Fig. 10, Lower Panel).

C. Conclusion

The results of this study show that AN1792 immunization of PDAPP mice possessing existing amyloid deposits slows and prevents progressive amyloid deposition and retard consequential neuropathologic changes in the aged PDAPP mouse brain. Immunizations with AN1792 essentially halted amyloid developing in structures that would normally succumb to amyloidosis. Thus, administration of A β peptide has therapeutic benefit in the treatment of AD.

IV. SCREEN OF A β FRAGMENTS

100 PDAPP mice age 9-11 months were immunized with 9 different regions of APP and A β to determine which epitopes convey the efficacious response. The 9 different immunogens and one control are injected i.p. as described above. The immunogens include four human A β peptide conjugates 1-12, 13-28, 32-42, 1-5, all coupled to sheep anti-mouse IgG via a cystine link; an APP polypeptide amino acids 592-695, aggregated human A β 1-40, and aggregated human A β 25-35, and aggregated rodent A β 42. Aggregated A β 42 and PBS were used as positive and negative controls, respectively. Ten mice were used per treatment group. Titers were monitored as above and mice are euthanized at the end of 4 months of injections. Histochemistry, A β levels, and toxicology analysis was determined post mortem.

A. Materials and Methods

1. Preparation of Immunogens

Preparation of coupled A β peptides: four human A β peptide conjugates (amino acid residues 1-5, 1-12, 13-28, and 33-42, each conjugated to sheep anti-mouse IgG) were prepared by coupling through an artificial cysteine added to the A β peptide using the crosslinking reagent sulfo-EMCS. The A β peptide derivatives were synthesized with the following final amino acid sequences. In each case, the location of the inserted cysteine residue is indicated by underlining. The A β 13-28 peptide derivative also had two glycine residues added prior to the carboxyl terminal cysteine as indicated.

A β 1-12 peptide	NH ₂ -DAEFRHDSGYEVC-COOH
A β 1-5 peptide	NH ₂ -DAEFRC-COOH
A β 33-42 peptide	NH ₂ -C-amino-heptanoic acid-GLMVGGVVIA-COOH
A β 13-28 peptide	Ac-NH-HH <u>Q</u> KL <u>V</u> FFAEDVGSNKGGC-COOH

To prepare for the coupling reaction, ten mg of sheep anti-mouse IgG (Jackson ImmunoResearch Laboratories) was dialyzed overnight against 10 mM sodium borate buffer, pH 8.5. The dialyzed antibody was then concentrated to a volume of 2 mL using an Amicon Centriprep tube. Ten mg sulfo-EMCS

[N (ϵ -maleimidocaproyloxy) succinimide] (Molecular Sciences Co.) was dissolved in one mL deionized water. A 40-fold molar excess of sulfo-EMCS was added dropwise with stirring to the sheep anti-mouse IgG and then the solution was stirred for an additional ten min. The activated sheep anti-mouse IgG was purified and buffer exchanged by passage over a 10 mL gel filtration column (Pierce Presto Column, obtained from Pierce Chemicals) equilibrated with 0.1 M NaPO₄, 5 mM EDTA, pH 6.5. Antibody containing fractions, identified by absorbance at 280 nm, were pooled and diluted to a concentration of approximately 1 mg/mL, using 1.4 mg per OD as the extinction coefficient. A 40-fold molar excess of A β peptide was dissolved in 20 mL of 10 mM NaPO₄, pH 8.0, with the exception of the A β 33-42 peptide for which 10 mg was first dissolved in 0.5 mL of DMSO and then diluted to 20 mL with the 10 mM NaPO₄ buffer. The peptide solutions were each added to 10 mL of activated sheep anti-mouse IgG and rocked at room temperature for 4 hr. The resulting conjugates were concentrated to a final volume of less than 10 mL using an Amicon Centriprep tube and then dialyzed

against PBS to buffer exchange the buffer and remove free peptide. The conjugates were passed through 0.22 μ m-pore size filters for sterilization and then aliquoted into fractions of 1 mg and stored frozen at -20°C. The concentrations of the conjugates were determined using the BCA protein assay (Pierce Chemicals) with horse IgG for the standard curve. Conjugation was documented by the molecular weight increase of the conjugated peptides relative to that of the activated sheep anti-mouse IgG. The A β 1-5 sheep anti-mouse conjugate was a pool of two conjugations, the rest were from a single preparation.

2. Preparation of aggregated A β peptides

Human 1-40 (AN1528; California Peptides Inc., Lot ME0541), human 1-42 (AN1792; California Peptides Inc., Lots ME0339 and ME0439), human 25-35, and rodent 1-42 (California Peptides Inc., Lot ME0218) peptides were freshly solubilized for the preparation of each set of injections from lyophilized powders that had been stored desiccated at -20°C. For this purpose, two mg of peptide were added to 0.9 ml of deionized water and the mixture was vortexed to generate a relatively uniform solution or suspension. Of the four, AN1528 was the only peptide soluble at this step. A 100 μ l aliquot of 10X PBS (1X PBS: 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5) was then added at which point AN1528 began to precipitate. The suspension was vortexed again and incubated overnight at 37°C for use the next day.

Preparation of the pBx6 protein: An expression plasmid encoding pBx6, a fusion protein consisting of the 100-amino acid bacteriophage MS-2 polymerase N-terminal leader sequence followed by amino acids 592-695 of APP (β APP) was constructed as described by Oltersdorf et al., J. Biol. Chem. 265, 4492-4497 (1990). The plasmid was transfected into E. coli and the protein was expressed after induction of the promoter. The bacteria were lysed in 8M urea and pBx6 was partially purified by preparative SDS PAGE. Fractions containing pBx6 were identified by Western blot using a rabbit anti-pBx6 polyclonal antibody, pooled, concentrated using an Amicon Centriprep tube and dialysed against PBS. The purity of the preparation, estimated by Coomassie Blue stained SDS PAGE, was approximately 5 to 10%.

B. Results and Discussion

1. Study Design

One hundred male and female, nine- to eleven-month old heterozygous PDAPP transgenic mice were obtained from Charles River Laboratory and Taconic Laboratory. The mice were sorted into ten groups to be immunized with different regions of A β or APP combined with Freund's adjuvant. Animals were distributed to match the gender, age, parentage and source of the animals within the groups as closely as possible. The immunogens included four A β peptides derived from the human sequence, 1-5, 1-12, 13-28, and 33-42, each conjugated to sheep anti-mouse IgG; four aggregated A β peptides, human 1-40 (AN1528), human 1-42 (AN1792), human 25-35, and rodent 1-42; and a fusion polypeptide, designated as pBx6, containing APP amino acid residues 592-695. A tenth group was immunized with PBS combined with adjuvant as a control.

For each immunization, 100 μ g of each A β peptide in 200 μ l PBS or 200 μ g of the APP derivative pBx6 in the same volume of PBS or PBS alone was emulsified 1:1 (vol:vol) with Complete Freund's adjuvant (CFA) in a final volume of 400 μ l for the first immunization, followed by a boost of the same amount of immunogen in Incomplete Freund's adjuvant (IFA) for the subsequent four doses and with PBS for the final dose. Immunizations were delivered intraperitoneally on a biweekly schedule for the first three doses, then on a monthly schedule thereafter. Animals were bled four to seven days following each immunization starting after the second dose for the measurement of antibody titers. Animals were euthanized approximately one week after the final dose.

2. A β and APP Levels in the Brain

Following about four months of immunization with the various A β peptides or the APP derivative, brains were removed from saline-perfused animals. One hemisphere was prepared for immunohistochemical analysis and the second was used for the quantitation of A β and APP levels. To measure the concentrations of various forms of beta amyloid peptide and amyloid precursor protein, the hemisphere was dissected and homogenates of the hippocampal, cortical, and cerebellar regions were prepared in 5 M guanidine. These were diluted and the level of amyloid or APP was quantitated by comparison to a series of dilutions of standards of A β peptide or APP of known concentrations in an ELISA format.

The median concentration of total A β for the control group immunized with PBS was 5.8-fold higher in the hippocampus than in the cortex (median of 24,318 ng/g hippocampal tissue compared to 4,221 ng/g for the cortex). The median level in the cerebellum of the control group (23.4 ng/g tissue) was about 1,000-fold lower than in the hippocampus. These levels are similar to those that we have previously reported for heterozygous PDAPP transgenic mice of this age (Johnson-Woods et al., 1997, *supra*).

For the cortex, a subset of treatment groups had median total A β and A β 1-42 levels which differed significantly from those of the control group ($p < 0.05$), those animals receiving AN1792, rodent A β 1-42 or the A β 1-5 peptide conjugate as shown in Fig. 11. The median levels of total A β were reduced by 75%, 79% and 61%, respectively, compared to the control for these treatment groups. There were no discernable correlations between A β -specific antibody titers and A β levels in the cortical region of the brain for any of the groups.

In the hippocampus, the median reduction of total A β associated with AN1792 treatment (46%, $p = 0.0543$) was not as great as that observed in the cortex (75%, $p = 0.0021$). However, the magnitude of the reduction was far greater in the hippocampus than in the cortex, a net reduction of 11,186 ng/g tissue in the hippocampus versus 3,171 ng/g tissue in the cortex. For groups of animals receiving rodent A β 1-42 or A β 1-5, the median total A β levels were reduced by 36% and 26%, respectively. However, given the small group sizes and the high variability of the amyloid peptide levels from animal to animal within both groups, these reductions were not significant. When the levels of A β 1-42 were measured in the hippocampus, none of the treatment-induced reductions reached significance. Thus, due to the smaller A β burden in the cortex, changes in this region are a more sensitive indicator of treatment effects. The changes in A β levels measured by ELISA in the cortex are similar, but not identical, to the results from the immunohistochemical analysis (see below).

Total A β was also measured in the cerebellum, a region typically minimally affected with AD pathology. None of the median A β concentrations of any of the groups immunized with the various A β peptides or the APP derivative differed from that of the control group in this region of the brain. This result suggests that non-pathological levels of A β are unaffected by treatment.

APP concentration was also determined by ELISA in the cortex and cerebellum from treated and control mice. Two different APP assays were utilized. The

first, designated APP- α /FL, recognizes both APP- α (α , the secreted form of APP which has been cleaved within the A β sequence), and full-length forms (FL) of APP, while the second recognizes only APP- α . In contrast to the treatment-associated diminution of A β in a subset of treatment groups, the levels of APP were unchanged in all of the treated compared to the control animals. These results indicate that the immunizations with A β peptides are not depleting APP; rather the treatment effect is specific to A β .

In summary, total A β and A β 1-42 levels were significantly reduced in the cortex by treatment with AN1792, rodent A β 1-42 or A β 1-5 conjugate. In the hippocampus, total A β was significantly reduced only by AN1792 treatment. No other treatment-associated changes in A β or APP levels in the hippocampal, cortical or cerebellar regions were significant.

2. Histochemical Analyses

Brains from a subset of six groups were prepared for immunohistochemical analysis, three groups immunized with the A β peptide conjugates A β 1-5, A β 1-12, and A β 13-28; two groups immunized with the full length A β aggregates AN1792 and AN1528 and the PBS-treated control group. The results of image analyses of the amyloid burden in brain sections from these groups are shown in Fig. 12. There were significant reductions of amyloid burden in the cortical regions of three of the treatment groups versus control animals. The greatest reduction of amyloid burden was observed in the group receiving AN1792 where the mean value was reduced by 97% ($p = 0.001$). Significant reductions were also observed for those animals treated with AN1528 (95%, $p = 0.005$) and the A β 1-5 peptide conjugate (67%, $p = 0.02$).

The results obtained by quantitation of total A β or A β 1-42 by ELISA and amyloid burden by image analysis differ to some extent. Treatment with AN1528 had a significant impact on the level of cortical amyloid burden when measured by quantitative image analysis but not on the concentration of total A β in the same region when measured by ELISA. The difference between these two results is likely to be due to the specificities of the assays. Image analysis measures only insoluble A β aggregated into plaques. In contrast, the ELISA measures all forms of A β , both soluble and insoluble, monomeric and aggregated. Since the disease pathology is thought to be associated with

the insoluble plaque-associated form of A β , the image analysis technique may have more sensitivity to reveal treatment effects. However since the ELISA is a more rapid and easier assay, it is very useful for screening purposes. Moreover it may reveal that the treatment-associated reduction of A β is greater for plaque-associated than total A β .

To determine if the A β -specific antibodies elicited by immunization in the treated animals reacted with deposited brain amyloid, a subset of the sections from the treated animals and the control mice were reacted with an antibody specific for mouse IgG. In contrast to the PBS group, A β -containing plaques were coated with endogenous IgG for animals immunized with the A β peptide conjugates A β 1-5, A β 1-12, and A β 13-28; and the full length A β aggregates AN1792 and AN1528. Brains from animals immunized with the other A β peptides or the APP peptide pBx6 were not analyzed by this assay.

3. Measurement of Antibody Titers

Mice were bled four to seven days following each immunization starting after the second immunization, for a total of five bleeds. Antibody titers were measured as A β 1-42-binding antibody using a sandwich ELISA with plastic multi-well plates coated with A β 1-42. As shown in Fig. 13, peak antibody titers were elicited following the fourth dose for those four vaccines which elicited the highest titers of AN1792-specific antibodies: AN1792 (peak GMT: 94,647), AN1528 (peak GMT: 88,231), A β 1-12 conjugate (peak GMT: 47,216) and rodent A β 1-42 (peak GMT: 10,766). Titers for these groups declined somewhat following the fifth and sixth doses. For the remaining five immunogens, peak titers were reached following the fifth or the sixth dose and these were of much lower magnitude than those of the four highest titer groups: A β 1-5 conjugate (peak GMT: 2,356), pBx6 (peak GMT: 1,986), A β 13-28 conjugate (peak GMT: 1,183), A β 33-42 conjugate (peak GMT: 658), A β 25-35 (peak GMT: 125). Antibody titers were also measured against the homologous peptides using the same ELISA sandwich format for a subset of the immunogens, those groups immunized with A β 1-5, A β 13-28, A β 25-35, A β 33-42 or rodent A β 1-42. These titers were about the same as those measured against A β 1-42 except for the rodent A β 1-42 immunogen in which case antibody titers against the homologous immunogen were about two-fold higher. The magnitude of the AN1792-specific antibody titer of individual animals or the mean values

of treatment groups did not correlate with efficacy measured as the reduction of A β in the cortex.

4. Lymphoproliferative Responses

A β -dependent lymphoproliferation was measured using spleen cells harvested approximately one week following the final, sixth, immunization. Freshly harvested cells, 105 per well, were cultured for 5 days in the presence of A β 1-40 at a concentration of 5 μ M for stimulation. Cells from a subset of seven of the ten groups were also cultured in the presence of the reverse peptide, A β 40-1. As a positive control, additional cells were cultured with the T cell mitogen, PHA, and, as a negative control, cells were cultured without added peptide.

Lymphocytes from a majority of the animals proliferated in response to PHA. There were no significant responses to the A β 40-1 reverse peptide. Cells from animals immunized with the larger aggregated A β peptides, AN1792, rodent A β 1-42 and AN1528 proliferated robustly when stimulated with A β 1-40 with the highest cpm in the recipients of AN1792. One animal in each of the groups immunized with A β 1-12 conjugate, A β 13-28 conjugate and A β 25-35 proliferated in response to A β 1-40. The remaining groups receiving A β 1-5 conjugate, A β 33-42 conjugate pBx6 or PBS had no animals with an A β -stimulated response. These results are summarized in Table 5 below.

Table 5			
Immunogen	Conjugate	A β Amino Acids	Responders
A β 1-5	Yes	5-mer	0/7
A β 1-12	Yes	12-mer	1/8
A β 13-28	Yes	16-mer	1/9
A β 25-35		11-mer	1/9
A β 33-42	Yes	10-mer	0/10
A β 1-40		40-mer	5/8
A β 1-42		42-mer	9/9
r A β 1-42		42-mer	8/8
pBx6			0/8
PBS		0-mer	0/8

These results show that AN1792 and AN1528 stimulate strong T cell responses, most likely of the CD4+ phenotype. The absence of an A β -specific T cell

response in animals immunized with A β 1-5 is not surprising since peptide epitopes recognized by CD4+ T cells are usually about 15 amino acids in length, although shorter peptides can sometimes function with less efficiency. Thus the majority of helper T cell epitopes for the four conjugate peptides are likely to reside in the IgG conjugate partner, not in the A β region. This hypothesis is supported by the very low incidence of proliferative responses for animals in each of these treatment groups. Since the A β 1-5 conjugate was effective at significantly reducing the level of A β in the brain, in the apparent absence of A β -specific T cells, the key effector immune response induced by immunization with this peptide appears to be antibody.

Lack of T-cell and low antibody response from fusion peptide pBx6, encompassing APP amino acids 592-695 including all of the A β residues may be due to the poor immunogenicity of this particular preparation. The poor immunogenicity of the A β 25-35 aggregate is likely due to the peptide being too small to be likely to contain a good T cell epitope to help the induction of an antibody response. If this peptide were conjugated to a carrier protein, it would probably be more immunogenic.

V. Preparation of Polyclonal Antibodies for Passive Protection

125 non-transgenic mice were immunized with A β , plus adjuvant, and euthanized at 4-5 months. Blood was collected from immunized mice. IgG was separated from other blood components. Antibody specific for the immunogen may be partially purified by affinity chromatography. An average of about 0.5-1 mg of immunogen-specific antibody is obtained per mouse, giving a total of 60-120mg.

VI. Passive Immunization with Antibodies to A β

Groups of 7-9 month old PDAPP mice each were injected with 0.5 mg in PBS of polyclonal anti-A β or specific anti-A β monoclonals as shown below. All antibody preparations were purified to have low endotoxin levels. Monoclonals can be prepared against a fragment by injecting the fragment or longer form of A β into a mouse, preparing hybridomas and screening the hybridomas for an antibody that specifically binds to a desired fragment of A β without binding to other nonoverlapping fragments of A β .

Table 6

Antibody	Epitope
2H3	A β 1-12
10D5	A β 1-12
266	A β 13-28
21F12	A β 33-42
Mouse polyclonal anti-human A β 42	Anti-Aggregated A β 42

Mice were injected ip as needed over a 4 month period to maintain a circulating antibody concentration measured by ELISA titer of greater than 1/1000 defined by ELISA to A β 42 or other immunogen. Titers were monitored as above and mice were euthanized at the end of 6 months of injections. Histochemistry, A β levels and toxicology were performed post mortem. Ten mice were used per group.

VII. Comparison of Different Adjuvants

This example compares CFA, alum, an oil-in water emulsion and MPL for capacity to stimulate an immune response.

A. Materials and Methods

1. Study Design

One hundred female Hartley strain six-week old guinea pigs, obtained from Elm Hill, were sorted into ten groups to be immunized with AN1792 or a palmitoylated derivative thereof combined with various adjuvants. Seven groups received injections of AN1792 (33 μ g unless otherwise specified) combined with a) PBS, b) Freund's adjuvant, c) MPL, d) squalene, e) MPL/squalene f) low dose alum, or g) high dose alum (300 μ g AN1792). Two groups received injections of a palmitoylated derivative of AN1792 (33 μ g) combined with a) PBS or b) squalene. A final, tenth group received PBS alone without antigen or additional adjuvant. For the group receiving Freund's adjuvant, the first dose was emulsified with CFA and the remaining four doses

with IFA. Antigen was administered at a dose of 33 μ g for all groups except the high dose alum group, which received 300 μ g of AN1792. Injections were administered intraperitoneally for CFA/IFA and intramuscularly in the hind limb quadriceps alternately on the right and left side for all other groups. The first three doses were given on a biweekly schedule followed by two doses at a monthly interval). Blood was drawn six to seven days following each immunization, starting after the second dose, for measurement of antibody titers.

2. Preparation of Immunogens

Two mg A β 42 (California Peptide, Lot ME0339) was added to 0.9 ml of deionized water and the mixture was vortexed to generate a relatively uniform suspension. A 100 μ l aliquot of 10X PBS (1X PBS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5) was added. The suspension was vortexed again and incubated overnight at 37°C for use the next day. Unused A β 1-42 was stored with desiccant as a lyophilized powder at -20°C.

A palmitoylated derivative of AN1792 was prepared by coupling palmitic anhydride, dissolved in dimethyl formamide, to the amino terminal residue of AN1792 prior to removal of the nascent peptide from the resin by treatment with hydrofluoric acid.

To prepare vaccine doses with Complete Freund's adjuvant (CFA) (group 2), 33 μ g of AN1792 in 200 μ l PBS was emulsified 1:1 (vol:vol) with CFA in a final volume of 400 μ l for the first immunization. For subsequent immunizations, the antigen was similarly emulsified with Incomplete Freund's adjuvant (IFA).

To prepare vaccine doses with MPL for groups 5 and 8, lyophilized powder (Ribi ImmunoChem Research, Inc., Hamilton, MT) was added to 0.2% aqueous triethylamine to a final concentration of 1 mg/ml and vortexed. The mixture was heated to 65 to 70°C for 30 sec to create a slightly opaque uniform suspension of micelles. The solution was freshly prepared for each set of injections. For each injection in group 5, 33 μ g of AN1792 in 16.5 μ l PBS, 50 μ g of MPL (50 μ l) and 162 μ l of PBS were mixed in a borosilicate tube immediately before use.

To prepare vaccine doses with the low oil-in-water emulsion, AN1792 in PBS was added to 5% squalene, 0.5% Tween 80, 0.5% Span 85 in PBS to reach a final single dose concentration of 33 μ g AN1792 in 250 μ l (group 6). The mixture was emulsified by passing through a two-chambered hand-held device 15 to 20 times until the

emulsion droplets appeared to be about equal in diameter to a 1.0 μm diameter standard latex bead when viewed under a microscope. The resulting suspension was opalescent, milky white. The emulsions were freshly prepared for each series of injections. For group 8, MPL in 0.2% triethylamine was added at a concentration of 50 μg per dose to the squalene and detergent mixture for emulsification as noted above. For the palmitoyl derivative (group 7), 33 μg per dose of palmitoyl-NH-A β 1-42 was added to squalene and vortexed. Tween 80 and Span 85 were then added with vortexing. This mixture was added to PBS to reach final concentrations of 5% squalene, 0.5% Tween 80, 0.5% Span 85 and the mixture was emulsified as noted above.

To prepare vaccine doses with alum (groups 9 and 10), AN1792 in PBS was added to Alhydrogel (aluminum hydroxide gel, Accurate, Westbury, NY) to reach concentrations of 33 μg (low dose, group 9) or 300 μg (high dose, group 10) AN1792 per 5 mg of alum in a final dose volume of 250 μl . The suspension was gently mixed for 4 hr at RT.

3. Measurement of Antibody Titers

Guinea pigs were bled six to seven days following immunization starting after the second immunization for a total of four bleeds. Antibody titers against A β 42 were measured by ELISA as described in General Materials and Methods.

4. Tissue Preparation

After about 14 weeks, all guinea pigs were administered CO₂. Cerebrospinal fluid was collected and the brains were removed and three brain regions (hippocampus, cortex and cerebellum) were dissected and used to measure the concentration of total A β protein using ELISA.

B. Results

1. Antibody Responses

There was a wide range in the potency of the various adjuvants when measured as the antibody response to AN1792 following immunization. As shown in Fig. 14, when AN1792 was administered in PBS, no antibody was detected following two or three immunizations and negligible responses were detected following the fourth and fifth doses with geometric mean titers (GMTs) of only about 45. The o/w emulsion

induced modest titers following the third dose (GMT 255) that were maintained following the fourth dose (GMT 301) and fell with the final dose (GMT 54). There was a clear antigen dose response for AN1792 bound to alum with 300 μ g being more immunogenic at all time points than 33 μ g. At the peak of the antibody response, following the fourth immunization, the difference between the two doses was 43% with GMTs of about 1940 (33 μ g) and 3400 (300 μ g). The antibody response to 33 μ g AN1792 plus MPL was very similar to that generated with almost a ten-fold higher dose of antigen (300 μ g) bound to alum. The addition of MPL to an o/w emulsion decreased the potency of the vaccine relative to that with MPL as the sole adjuvant by as much as 75%. A palmitoylated derivative of AN1792 was completely non-immunogenic when administered in PBS and gave modest titers when presented in an o/w emulsion with GMTs of 340 and 105 for the third and fourth bleeds. The highest antibody titers were generated with Freund's adjuvant with a peak GMT of about 87,000, a value almost 30-fold greater than the GMTs of the next two most potent vaccines, MPL and high dose AN1792/alum.

The most promising adjuvants identified in this study are MPL and alum. Of these two, MPL appears preferable because a 10-fold lower antigen dose was required to generate the same antibody response as obtained with alum. The response can be increased by increasing the dose of antigen and /or adjuvant and by optimizing the immunization schedule. The o/w emulsion was a very weak adjuvant for AN1792 and adding an o/w emulsion to MPL adjuvant diminished the intrinsic adjuvant activity of MPL alone.

2. A β Levels In The Brain

At about 14 weeks the guinea pigs were deeply anesthetized, the cerebrospinal fluid (CSF) was drawn and brains were excised from animals in a subset of the groups, those immunized with Freund's adjuvant (group 2), MPL (group 5), alum with a high dose, 300 μ g, of AN1792 (group 10) and the PBS immunized control group (group 3). To measure the level of A β peptide, one hemisphere was dissected and homogenates of the hippocampal, cortical, and cerebellar regions were prepared in 5 M guanidine. These were diluted and quantitated by comparison to a series of dilutions of A β standard protein of known concentrations in an ELISA format. The levels of A β protein in the hippocampus, the cortex and the cerebellum were very similar for all four groups despite the wide range of antibody responses to A β elicited by these vaccines. Mean A β levels of

Table 7

Experimental Design of Study 010					
Group	N ^a	Adjuvant ^b	Dose	Antigen	Dose (µg)
1	10	MPL	12.5 µg	AN1792	33
2	10	MPL	25 µg	AN1792	33
3	10	MPL	50 µg	AN1792	33
4	13	MPL	125 µg	AN1792	33
5	13	MPL	50 µg	AN1792	150
6	13	MPL	50 µg	AN1528	33
7	10	PBS		AN1792	33
8	10	PBS		None	
9	10	Squalene emulsified	5%	AN1792	33
10	10	Squalene admixed	5%	AN1792	33
11	10	Alum	2 mg	AN1792	33
12	13	MPL + Alum	50 µg/2 mg	AN1792	33
13	10	QS-21	5 µg	AN1792	33
14	10	QS-21	10 µg	AN1792	33
15	10	QS-21	25 AN1792	AN1792	33
16	13	QS-21	25 AN1792	AN1792	150
17	13	QS-21	25 AN1792	AN1528	33
18	13	QS-21 + MPL	25 µg/50 µg	AN1792	33
19	13	QS-21 + Alum	25 µg/2 mg	AN1792	33

Footnotes:

^a Number of mice in each group at the initiation of the experiment.^b The adjuvants are noted. The buffer for all these formulations was PBS. For group 8, there was no adjuvant and no antigen.

5

The ELISA titers of antibodies against A β 42 in each group are shown in Table 8 below.

Table 8.

Geometric Mean Antibody Titers					
Week of Bleed					
Treatment Group	2.9	5.0	8.7	12.9	16.7
1	248	1797	2577	6180	4177
2	598	3114	3984	5287	6878
3	1372	5000	7159	12333	12781
4	1278	20791	14368	20097	25631
5	3288	26242	13229	9315	23742
6	61	2536	2301	1442	4504
7	37	395	484	972	2149
8	25	25	25	25	25
9	25	183	744	952	1823
10	25	89	311	513	817
11	29	708	2618	2165	3666
12	198	1458	1079	612	797
13	38	433	566	1080	626
14	104	541	3247	1609	838
15	212	2630	2472	1224	1496
16	183	2616	6680	2085	1631
17	28	201	375	222	1540
18	31699	15544	23095	6412	9059
19	63	243	554	299	441

- 5 The table shows that the highest titers were obtained for groups 4, 5 and 18, in which the adjuvants were 125 μ g MPL, 50 μ g MPL and QS-21 plus MPL.

IX. Therapeutic Efficacy of Different Adjuvants

A therapeutic efficacy study was conducted in PDAPP transgenic mice with a set of adjuvants suitable for use in humans to determine their ability to potentiate immune responses to A β and to induce the immune-mediated clearance of amyloid deposits in the brain.

One hundred eighty male and female, 7.5- to 8.5-month old heterozygous PDAPP transgenic mice were obtained from Charles River Laboratories. The mice were

sorted into nine groups containing 15 to 23 animals per group to be immunized with AN1792 or AN1528 combined with various adjuvants. Animals were distributed to match the gender, age, and parentage of the animals within the groups as closely as possible. The adjuvants included alum, MPL, and QS-21, each combined with both
5 antigens, and Freund's adjuvant (FA) combined with only AN1792. An additional group was immunized with AN1792 formulated in PBS buffer plus the preservative thimerosal without adjuvant. A ninth group was immunized with PBS alone as a negative control.

Preparation of aggregated A β peptides: human A β 1-40 (AN1528; California Peptides Inc., Napa, CA; Lot ME0541) and human A β 1-42 (AN1792; California Peptides Inc., Lot ME0439) peptides were freshly solubilized for the
10 preparation of each set of injections from lyophilized powders that had been stored desiccated at -20°C. For this purpose, two mg of peptide were added to 0.9 ml of deionized water and the mixture was vortexed to generate a relatively uniform solution or suspension. AN1528 was soluble at this step, in contrast to AN1792. A 100 μ l aliquot
15 of 10X PBS (1X PBS: 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5) was then added at which point AN1528 began to precipitate. The suspensions were vortexed again and incubated overnight at 37°C for use the next day.

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To prepare vaccine doses with alum (Groups 1 and 5), A β peptide in PBS was added to Alhydrogel (two percent aqueous aluminum hydroxide gel, Sargeant, Inc., Clifton, NJ) to reach concentrations of 100 μ g A β peptide per 1 mg of alum. 10X PBS was added to a final dose volume of 200 μ l in 1X PBS. The suspension was then gently mixed for approximately 4 hr at RT prior to injection.

To prepare vaccine doses for with MPL (Groups 2 and 6), lyophilized powder (Ribi ImmunoChem Research, Inc., Hamilton, MT; Lot 67039-E0896B) was
25 added to 0.2% aqueous triethylamine to a final concentration of 1 mg/ml and vortexed. The mixture was heated to 65 to 70°C for 30 sec to create a slightly opaque uniform suspension of micelles. The solution was stored at 4°C. For each set of injections, 100 μ g of peptide per dose in 50 μ l PBS, 50 μ g of MPL per dose (50 μ l) and 100 μ l of PBS per dose were mixed in a borosilicate tube immediately before use.

To prepare vaccine doses with QS-21 (Groups 3 and 7), lyophilized powder (Aquila, Framingham, MA; Lot A7018R) was added to PBS, pH 6.6-6.7 to a final concentration of 1 mg/ml and vortexed. The solution was stored at -20°C. For each set of injections, 100 μ g of peptide per dose in 50 μ l PBS, 25 μ g of QS-21 per dose in 25

µl PBS and 125 µl of PBS per dose were mixed in a borosilicate tube immediately before use.

To prepare vaccine doses with Freund's Adjuvant (Group 4), 100 µg of AN1792 in 200 µl PBS was emulsified 1:1 (vol:vol) with Complete Freund's Adjuvant (CFA) in a final volume of 400 µl for the first immunization. For subsequent immunizations, the antigen was similarly emulsified with Incomplete Freund's Adjuvant (IFA). For the vaccines containing the adjuvants alum, MPL or QS-21, 100 µg per dose of AN1792 or AN1528 was combined with alum (1 mg per dose) or MPL (50 µg per dose) or QS-21 (25 µg per dose) in a final volume of 200 µl PBS and delivered by subcutaneous inoculation on the back between the shoulder blades. For the group receiving FA, 100 µg of AN1792 was emulsified 1:1 (vol:vol) with Complete Freund's adjuvant (CFA) in a final volume of 400 µl and delivered intraperitoneally for the first immunization, followed by a boost of the same amount of immunogen in Incomplete Freund's adjuvant (IFA) for the subsequent five doses. For the group receiving AN1792 without adjuvant, 10 µg AN1792 was combined with 5 µg thimerosal in a final volume of 50 µl PBS and delivered subcutaneously. The ninth, control group received only 200 µl PBS delivered subcutaneously. Immunizations were given on a biweekly schedule for the first three doses, then on a monthly schedule thereafter on days 0, 16, 28, 56, 85 and 112. Animals were bled six to seven days following each immunization starting after the second dose for the measurement of antibody titers. Animals were euthanized approximately one week after the final dose. Outcomes were measured by ELISA assay of Aβ and APP levels in brain and by immunohistochemical evaluation of the presence of amyloid plaques in brain sections. In addition, Aβ-specific antibody titers, and Aβ-dependent proliferative and cytokine responses were determined.

Table 9 shows that the highest antibody titers to Aβ1-42 were elicited with FA and AN1792, titers which peaked following the fourth immunization (peak GMT: 75,386) and then declined by 59% after the final, sixth immunization. The peak mean titer elicited by MPL with AN1792 was 62% lower than that generated with FA (peak GMT: 28,867) and was also reached early in the immunization scheme, after 3 doses, followed by a decline to 28% of the peak value after the sixth immunization. The peak mean titer generated with QS-21 combined with AN1792 (GMT: 1,511) was about 5-fold lower than obtained with MPL. In addition, the kinetics of the response were slower, since an additional immunization was required to reach the peak response. Titers

generated by alum-bound AN1792 were marginally greater than those obtained with QS-21 and the response kinetics were more rapid. For AN1792 delivered in PBS with thimerosal the frequency and size of titers were barely greater than that for PBS alone. The peak titers generated with MPL and AN1528 (peak GMT 3099) were about 9-fold
5 lower than those with AN1792. Alum-bound AN1528 was very poorly immunogenic with low titers generated in only some of the animals. No antibody responses were observed in the control animals immunized with PBS alone.

Table 9

Geometric Mean Antibody Titers ^a					
Week of Bleed					
Treatment	3.3	5.0	9.0	13.0	17.0
Alum/ AN1792	102 (12/21) ^b	1,081 (17/20)	2,366 (21/21)	1,083 (19/21)	572 (18/21)
MPL/ AN1792	6241 (21/21)	28,867 (21/21)	1,1242 (21/21)	5,665 (20/20)	8,204 (20/20)
QS-21/ AN1792	30 (1/20)	227 (10/19)	327 (10/19)	1,511 (17/18)	1,188 (14/18)
CFA/ AN1792	10,076 (15/15)	61,279 (15/15)	75,386 (15/15)	41,628 (15/15)	30,574 (15/15)
Alum/ AN1528	25 (0/21)	33 (1/21)	39 (3/20)	37 (1/20)	31 (2/20)
MPL/ AN1528	184 (15/21)	2,591 (20/21)	1,653 (21/21)	1,156 (20/20)	3,099 (20/20)
QS-21/ AN1528	29 (1/22)	221 (13/22)	51 (4/22)	820 (20/22)	2,994 (21/22)
PBS plus Thimerosal	25 (0/16)	33 (2/16)	39 (4/16)	37 (3/16)	47 (4/16)
PBS	25 (0/16)	25 (0/16)	25 (0/15)	25 (0/12)	25 (0/16)

Footnotes:

^a Geometric mean antibody titers measured against A β 1-42^b Number of responders per group

Sub D4

The results of AN1792 or AN1528 treatment with various adjuvants, or thimerosal on cortical amyloid burden in 12-month old mice determined by ELISA are shown in Fig. 15. In PBS control PDAPP mice, the median level of total A β in the cortex at 12 months was 1,817 ng/g. Notably reduced levels of A β were observed in mice treated with AN1792 plus CFA/IFA, AN1792 plus alum, AN1792 plus MPL and QS-21 plus AN1792. The reduction reached statistical significance ($p < 0.05$) only for AN1792 plus CFA/IFA. However, as shown in Examples I and III, the effects of immunization in reducing A β levels become substantially greater in 15 month and 18 month old mice. Thus, it is expected that at least the AN1792 plus alum, AN1792 plus MPL and AN1792 plus QS-21 compositions will achieve statistical significance in treatment of older mice. By contrast, the AN1792 plus the preservative thimerosal showed a median level of A β about the same as that in the PBS treated mice. Similar results were obtained when cortical levels of A β 42 were compared. The median level of A β 42 in PBS controls was 1624 ng/g. Notably reduced median levels of 403, 1149, 620 and 714 were observed in the mice treated with AN1792 plus CFA/IFA, AN1792 plus alum, AN1792 plus MPL and AN1792 plus QS-21 respectively, with the reduction achieving statistical significance ($p = 0.05$) for the AN1792 CFA/IFA treatment group. The median level in the AN1792 thimerosal treated mice was 1619 ng/g A β 42.

X. Toxicity Analysis

Tissues were collected for histopathologic examination at the termination of studies described in Examples 2, 3 and 7. In addition, hematology and clinical chemistry were performed on terminal blood samples from Examples 3 and 7. Most of the major organs were evaluated, including brain, pulmonary, lymphoid, gastrointestinal, liver, kidney, adrenal and gonads. Although sporadic lesions were observed in the study animals, there were no obvious differences, either in tissues affected or lesion severity, between AN1792 treated and untreated animals. There were no unique histopathological lesions noted in AN-1528-immunized animals compared to PBS-treated or untreated animals. There were also no differences in the clinical chemistry profile between adjuvant groups and the PBS treated animals in Example 7. Although there were significant increases in several of the hematology parameters between animals treated with AN1792 and Freund's adjuvant in Example 7 relative to PBS treated animals, these type of effects are expected from Freund's adjuvant treatment and the accompanying

peritonitis and do not indicate any adverse effects from AN1792 treatment. Although not part of the toxicological evaluation, PDAPP mouse brain pathology was extensively examined as part of the efficacy endpoints. No sign of treatment related adverse effect on brain morphology was noted in any of the studies. These results indicate that AN1792 treatment is well tolerated and at least substantially free of side effects.

XI. Therapeutic Treatment with Anti-A β antibodies

This examples tests the capacity of various monoclonal and polyclonal antibodies to A β to inhibit accumulation of A β in the brain of heterozygotic transgenic mice.

1. Study Design

Sixty male and female, heterozygous PDAPP transgenic mice, 8.5 to 10.5 months of age were obtained from Charles River Laboratory. The mice were sorted into six groups to be treated with various antibodies directed to A β . Animals were distributed to match the gender, age, parentage and source of the animals within the groups as closely as possible. As shown in Table 10, the antibodies included four murine A β -specific monoclonal antibodies, 2H3 (directed to A β residues 1-12), 10D5 (directed to A β residues 1-16), 266 (directed to A β residues 13-28 and binds to monomeric but not to aggregated AN1792), 21F12 (directed to A β residues 33-42). A fifth group was treated with an A β -specific polyclonal antibody fraction (raised by immunization with aggregated AN1792). The negative control group received the diluent, PBS, alone without antibody.

The monoclonal antibodies were injected at a dose of about 10 mg/kg (assuming that the mice weighed 50 g). Injections were administered intraperitoneally every seven days on average to maintain anti-A β titers above 1000. Although lower titers were measured for mAb 266 since it does not bind well to the aggregated AN1792 used as the capture antigen in the assay, the same dosing schedule was maintained for this group. The group receiving monoclonal antibody 2H3 was discontinued within the first three weeks since the antibody was cleared too rapidly in vivo. Animals were bled prior to each dosing for the measurement of antibody titers. Treatment was continued over a six-month period for a total of 196 days. Animals were euthanized one week after the final dose.

Table 10

EXPERIMENTAL DESIGN OF STUDY 006				
Treatment Group	N ^a	Treatment Antibody	Antibody Specificity	Antibody Isotype
1	9	none (PBS alone)	NA ^b	NA
2	10	Polyclonal	A β 1-42	mixed
3	0	mAb ^c 2H3	A β 1-12	IgG1
4	8	mAb 10D5	A β 1-16	IgG1
5	6	mAb 266	A β 13-28	IgG1
6	8	mAb 21F12	A β 33-42	IgG2a

Footnotes

- a. Number of mice in group at termination of the experiment. All groups started with 10 animals per group.
 b. NA: not applicable
 c. mAb: monoclonal antibody

2. Materials and Methods

a. Preparation of the Antibodies

The anti-A β polyclonal antibody was prepared from blood collected from two groups of animals. The first group consisted of 100 female Swiss Webster mice, 6 to 8 weeks of age. They were immunized on days 0, 15, and 29 with 100 μ g of AN1792 combined with CFA/IFA. A fourth injection was given on day 36 with one-half the dose of AN1792. Animals were exsanguinated upon sacrifice at day 42, serum was prepared and the sera were pooled to create a total of 64 ml. The second group consisted of 24 female mice isogenic with the PDAPP mice but nontransgenic for the human APP gene, 6 to 9 weeks of age. They were immunized on days 0, 14, 28 and 56 with 100 μ g of AN1792 combined with CFA/IFA. These animals were also exsanguinated upon sacrifice at day 63, serum was prepared and pooled for a total of 14 ml. The two lots of sera were pooled. The antibody fraction was purified using two sequential rounds of precipitation with 50% saturated ammonium sulfate. The final precipitate was dialyzed against PBS and tested for endotoxin. The level of endotoxin was less than 1 EU/mg.

The anti-A β monoclonal antibodies were prepared from ascities fluid. The fluid was first delipidated by the addition of concentrated sodium dextran sulfate to ice-cold ascites fluid by stirring on ice to reach a final concentration of 0.238%.

Concentrated CaCl₂ was then added with stirring to reach a final concentration of 64mM.

5 This solution was centrifuged at 10,000 x g and the pellet was discarded. The supernatant was stirred on ice with an equal volume of saturated ammonium sulfate added dropwise. The solution was centrifuged again at 10,000 x g and the supernatant was discarded. The pellet was resuspended and dialyzed against 20 mM Tris-HCl, 0.4 M NaCl, pH 7.5. This fraction was applied to a Pharmacia FPLC Sepharose Q Column and eluted with a reverse
10 gradient from 0.4 M to 0.275 M NaCl in 20 mM Tris-HCl, pH 7.5.

The antibody peak was identified by absorbance at 280 nm and appropriate fractions were pooled. The purified antibody preparation was characterized by measuring the protein concentration using the BCA method and the purity using SDS-PAGE. The pool was also tested for endotoxin. The level of endotoxin was less than 1 EU/mg. titers,
15 titers less than 100 were arbitrarily assigned a titer value of 25.

3. A β and APP Levels in the Brain:

Following about six months of treatment with the various anti-A β antibody preparations, brains were removed from the animals following saline perfusion. One
20 hemisphere was prepared for immunohistochemical analysis and the second was used for the quantitation of A β and APP levels. To measure the concentrations of various forms of beta amyloid peptide and amyloid precursor protein (APP), the hemisphere was dissected and homogenates of the hippocampal, cortical, and cerebellar regions were prepared in 5M guanidine. These were serially diluted and the level of amyloid peptide
25 or APP was quantitated by comparison to a series of dilutions of standards of A β peptide or APP of known concentrations in an ELISA format.

The levels of total A β and of A β 1-42 measured by ELISA in homogenates of the cortex, and the hippocampus and the level of total A β in the cerebellum are shown in Tables 11, 12, and 13, respectively. The median concentration of total A β for the
30 control group, inoculated with PBS, was 3.6-fold higher in the hippocampus than in the cortex (median of 63,389 ng/g hippocampal tissue compared to 17,818 ng/g for the cortex). The median level in the cerebellum of the control group (30.6 ng/g tissue) was more than 2,000-fold lower than in the hippocampus. These levels are similar to those

that we have previously reported for heterozygous PDAPP transgenic mice of this age (Johnson-Woods et al., 1997).

For the cortex, one treatment group had a median A β level, measured as A β 1-42, which differed significantly from that of the control group ($p < 0.05$), those
5 animals receiving the polyclonal anti-A β antibody as shown in Table 11. The median level of A β 1-42 was reduced by 65%, compared to the control for this treatment group. The median levels of A β 1-42 were also significantly reduced by 55% compared to the control in one additional treatment group, those animals dosed with the mAb 10D5 ($p = 0.0433$).

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Table 11

CORTEX

Treatment Group	N ^a	Medians						Means	
		Total A β			A β 42			Total A β	A β 42
		LISA value ^b	P value ^c	% Change	LISA value	P value	% Change	ELISA value	ELISA value
PBS	9	17818	NA ^d	NA	13802	NA	NA	16150+/-7456 ^e	12621+/-5738
Polyclonal anti-A β 42	10	6160	0.0055	-65	4892	0.0071	-65	5912+/-4492	4454+/-3347
mAb 10D5	8	7915	0.1019	-56	6214	0.0433	-55	9695+/-6929	6943+/-3351
mAb 266	6	9144	0.1255	-49	8481	0.1255	-39	9204+/-9293	7489+/-6921
mAb 21F12	8	15158	0.2898	-15	13578	0.7003	-2	12481+/-7082	11005+/-6324

Footnotes:

f. Number of animals per group at the end of the experiment

g. ng/g tissue

h. Mann Whitney analysis

i. NA: not applicable

j. Standard Deviation

In the hippocampus, the median percent reduction of total A β associated with treatment with polyclonal anti-A β antibody (50%, $p = 0.0055$) was not as great as that observed in the cortex (65%) (Table 12). However, the absolute magnitude of the reduction was almost 3-fold greater in the hippocampus than in the cortex, a net reduction of 31,683 ng/g tissue in the hippocampus versus 11,658 ng/g tissue in the cortex. When measured as the level of the more amyloidogenic form of A β , A β 1-42, rather than as total A β , the reduction achieved with the polyclonal antibody was significant ($p = 0.0025$). The median levels in groups treated with the mAbs 10D5 and 266 were reduced by 33% and 21%, respectively.

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Table 12

HIPPOCAMPUS												
Treatment Group	N ^a	Medians						Means				
		Total A β			A β 42			Total A β		A β 42		
		ELISA value ^b	P value ^c	% Change	ELISA value	P value	% Change	ELISA value		ELISA value		
PBS	9	63389	NA ^d	NA	54429	NA	NA	58351+/-13308 ^e		52801+/-14701		
Polyclonal anti-A β 42	10	31706	0.0055	-50	27127	0.0025	-50	30058+/-22454		24853+/-18262		
mAb 10D5	8	46779	0.0675	-26	36290	0.0543	-33	44581+/-18632		36465+/-17146		
mAb 266	6	48689	0.0990	-23	43034	0.0990	-21	36419+/-27304		32919+/-25372		
mAb 21F12	8	51563	0.7728	-19	47961	0.8099	-12	57327+/-28927		50305+/-23927		

Footnotes:

a. Number of animals per group at the end of the experiment

b. ng/g tissue

c. Mann Whitney analysis

d. NA: not applicable

e. Standard Deviation

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 5 *10D5* Total A β was also measured in the cerebellum (Table 13). Those groups dosed with the polyclonal anti-A β and the 266 antibody showed significant reductions of the levels of total A β (43% and 46%, $p = 0.0033$ and $p = 0.0184$, respectively) and that group treated with 10D5 had a near significant reduction (29%, $p = 0.0675$).

Table 13

<u>CEREBELLUM</u>					
Treatment Group	N ^a	Medians			Means
		Total A β			Total A β
		ELISA value ^b	P value ^c	% Change	ELISA value
PBS	9	30.64	NA ^d	NA	40.00+/-31.89 ^e
Polyclonal anti-A β 42	10	17.61	0.0033	-43	18.15+/-4.36
mAb 10D5	8	21.68	0.0675	-29	27.29+/-19.43
mAb 266	6	16.59	0.0184	-46	19.59+/-6.59
mAb 21F12	8	29.80	>0.9999	-3	32.88+/-9.90

Footnotes:

a. Number of animals per group at the end of the experiment

10 b. ng/g tissue

c. Mann Whitney analysis

d. NA: not applicable

e. Standard Deviation

15 APP concentration was also determined by ELISA in the cortex and cerebellum from antibody-treated and control, PBS-treated mice. Two different APP assays were utilized. The first, designated APP- α /FL, recognizes both APP-alpha (α , the secreted form of APP which has been cleaved within the A β sequence), and full-length

forms (FL) of APP, while the second recognizes only APP- α . In contrast to the treatment-associated diminution of A β in a subset of treatment groups, the levels of APP were virtually unchanged in all of the treated compared to the control animals. These results indicate that the immunizations with A β antibodies deplete A β without depleting APP.

In summary, A β levels were significantly reduced in the cortex, hippocampus and cerebellum in animals treated with the polyclonal antibody raised against AN1792. To a lesser extent monoclonal antibodies to the amino terminal region of A β 1-42, specifically amino acids 1-16 and 13-28 also showed significant treatment effects.

4. Histochemical Analyses:

The morphology of A β -immunoreactive plaques in subsets of brains from mice in the PBS, polyclonal A β 42, 21F12, 266 and 10D5 treatment groups was qualitatively compared to that of previous studies in which standard immunization procedures with A β 42 were followed.

The largest alteration in both the extent and appearance of amyloid plaques occurred in the animals immunized with the polyclonal A β 42 antibody. The reduction of amyloid load, eroded plaque morphology and cell-associated A β immunoreactivity closely resembled effects produced by the standard immunization procedure. These observations support the ELISA results in which significant reductions in both total A β and A β 42 were achieved by administration of the polyclonal A β 42 antibody.

In similar qualitative evaluations, amyloid plaques in the 10D5 group were also reduced in number and appearance, with some evidence of cell-associated A β immunoreactivity. Major differences were not seen when the 21F12 and 266 groups were compared with the PBS controls.

5. Measurement of Antibody Titers:

A subset of three randomly chosen mice from each group were bled just prior to each intraperitoneal inoculation, for a total of 30 bleeds. Antibody titers were measured as A β 1-42-binding antibody using a sandwich ELISA with plastic multi-well plates coated with A β 1-42 as described in detail in the General Materials and Methods. Mean titers for each bleed are shown in Figures 16-18 for the polyclonal antibody and the

monoclonals 10D5 and 21F12, respectively. Titers averaged about 1000 over this time period for the polyclonal antibody preparation and were slightly above this level for the 10D5- and 21F12-treated animals..

6. Lymphoproliferative Responses:

A β -dependent lymphoproliferation was measured using spleen cells harvested eight days following the final antibody infusion. Freshly harvested cells, 10⁵ per well, were cultured for 5 days in the presence of A β 1-40 at a concentration of 5 μ M for stimulation. As a positive control, additional cells were cultured with the T cell mitogen, PHA, and, as a negative control, cells were cultured without added peptide.

Splenocytes from aged PDAPP mice passively immunized with various anti-A β antibodies were stimulated *in vitro* with AN1792 and proliferative and cytokine responses were measured. The purpose of these assays was to determine if passive immunization facilitated antigen presentation, and thus priming of T cell responses specific for AN1792. No AN1792-specific proliferative or cytokine responses were observed in mice passively immunized with the anti-A β antibodies.

XII. Prevention and Treatment of Subjects

A single-dose phase I trial is performed to determine safety. A therapeutic agent is administered in increasing dosages to different patients starting from about 0.01 the level of presumed efficacy, and increasing by a factor of three until a level of about 10 times the effective mouse dosage is reached.

A phase II trial is performed to determine therapeutic efficacy. Patients with early to mid Alzheimer's Disease defined using Alzheimer's disease and Related Disorders Association (ADRDA) criteria for probable AD are selected. Suitable patients score in the 12-26 range on the Mini-Mental State Exam (MMSE). Other selection criteria are that patients are likely to survive the duration of the study and lack complicating issues such as use of concomitant medications that may interfere. Baseline evaluations of patient function are made using classic psychometric measures, such as the MMSE, and the ADAS, which is a comprehensive scale for evaluating patients with Alzheimer's Disease status and function. These psychometric scales provide a measure of progression of the Alzheimer's condition. Suitable qualitative life scales can also be used to monitor treatment. Disease progression can also be monitored by MRI. Blood profiles

of patients can also be monitored including assays of immunogen-specific antibodies and T-cells responses.

Following baseline measures, patients begin receiving treatment. They are randomized and treated with either therapeutic agent or placebo in a blinded fashion.

- 5 Patients are monitored at least every six months. Efficacy is determined by a significant reduction in progression of a treatment group relative to a placebo group.

A second phase II trial is performed to evaluate conversion of patients from non-Alzheimer's Disease early memory loss, sometimes referred to as age-associated memory impairment (AAMI), or mild cognitive impairment (MCI), to
10 probable Alzheimer's disease as defined as by ADRDA criteria. Patients with high risk for conversion to Alzheimer's Disease are selected from a non-clinical population by screening reference populations for early signs of memory loss or other difficulties associated with pre-Alzheimer's symptomatology, a family history of Alzheimer's Disease, genetic risk factors, age, sex, and other features found to predict high-risk for
15 Alzheimer's Disease. Baseline scores on suitable metrics including the MMSE and the ADAS together with other metrics designed to evaluate a more normal population are collected. These patient populations are divided into suitable groups with placebo comparison against dosing alternatives with the agent. These patient populations are followed at intervals of about six months, and the endpoint for each patient is whether or
20 not he or she converts to probable Alzheimer's Disease as defined by ADRDA criteria at the end of the observation.

XIII. General Materials and Methods

1. Measurement of Antibody Titers

25 Mice were bled by making a small nick in the tail vein and collecting about 200 μ l of blood into a microfuge tube. Guinea pigs were bled by first shaving the back hock area and then using an 18 gauge needle to nick the metatarsal vein and collecting the blood into microfuge tubes. Blood was allowed to clot for one hr at room temperature (RT), vortexed, then centrifuged at 14,000 x g for 10 min to separate the clot
30 from the serum. Serum was then transferred to a clean microfuge tube and stored at 4°C until titrated.

Antibody titers were measured by ELISA. 96-well microtiter plates (Costar EIA plates) were coated with 100 μ l of a solution containing either 10 μ g/ml

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either A β 42 or SAPP or other antigens as noted in each of the individual reports in Well Coating Buffer (0.1 M sodium phosphate, pH 8.5, 0.1% sodium azide) and held overnight at RT. The wells were aspirated and sera were added to the wells starting at a 1/100 dilution in Specimen Diluent (0.014 M sodium phosphate, pH 7.4, 0.15 M NaCl, 0.6% bovine serum albumin, 0.05% thimerosal). Seven serial dilutions of the samples were made directly in the plates in three-fold steps to reach a final dilution of 1/218,700. The dilutions were incubated in the coated-plate wells for one hr at RT. The plates were then washed four times with PBS containing 0.05% Tween 20. The second antibody, a goat anti-mouse Ig conjugated to horseradish peroxidase (obtained from Boehringer Mannheim), was added to the wells as 100 μ l of a 1/3000 dilution in Specimen Diluent and incubated for one hr at RT. Plates were again washed four times in PBS, Tween 20. To develop the chromogen, 100 μ l of Slow TMB (3,3',5,5'-tetramethyl benzidine obtained from Pierce Chemicals) was added to each well and incubated for 15 min at RT. The reaction was stopped by the addition of 25 μ l of 2 M H₂SO₄. The color intensity was then read on a Molecular Devices Vmax at (450 nm - 650 nm).

Titers were defined as the reciprocal of the dilution of serum giving one half the maximum OD. Maximal OD was generally taken from an initial 1/100 dilution, except in cases with very high titers, in which case a higher initial dilution was necessary to establish the maximal OD. If the 50% point fell between two dilutions, a linear extrapolation was made to calculate the final titer. To calculate geometric mean antibody titers, titers less than 100 were arbitrarily assigned a titer value of 25.

2. Lymphocyte proliferation assay

Mice were anesthetized with isoflurane. Spleens were removed and rinsed twice with 5 ml PBS containing 10% heat-inactivated fetal bovine serum (PBS-FBS) and then homogenized in a 50° Centricon unit (Dako A/S, Denmark) in 1.5 ml PBS-FBS for 10 sec at 100 rpm in a Medimachine (Dako) followed by filtration through a 100 micron pore size nylon mesh. Splenocytes were washed once with 15 ml PBS-FBS, then pelleted by centrifugation at 200 x g for 5 min. Red blood cells were lysed by resuspending the pellet in 5 mL buffer containing 0.15 M NH₄Cl, 1 M KHCO₃, 0.1 M NaEDTA, pH 7.4 for five min at RT. Leukocytes were then washed as above. Freshly isolated spleen cells (10⁵ cells per well) were cultured in triplicate sets in 96-well U-bottomed tissue culture-treated microtiter plates (Corning, Cambridge, MA) in RPMI 1640 medium (JRH

Biosciences, Lenexa, KS) supplemented with 2.05 mM L glutamine, 1% Penicillin/Streptomycin, and 10% heat-inactivated FBS, for 96 hr at 37°C. Various A β peptides, A β 1-16, A β 1-40, A β 1-42 or A β 40-1 reverse sequence protein were also added at doses ranging from 5 to 0.18 micromolar in four steps. Cells in control wells were
5 cultured with Concanavalin A (Con A) (Sigma, cat. # C-5275, at 1 microgram/ml) without added protein. Cells were pulsed for the final 24 hr with 3H-thymidine (1 μ Ci/well obtained from Amersham Corp., Arlington Heights IL). Cells were then harvested onto UniFilter plates and counted in a Top Count Microplate Scintillation Counter (Packard Instruments, Downers Grove, IL). Results are expressed as counts per
10 minute (cpm) of radioactivity incorporated into insoluble macromolecules.

4. Brain Tissue Preparation

After euthanasia, the brains were removed and one hemisphere was prepared for immunohistochemical analysis, while three brain regions (hippocampus,
15 cortex and cerebellum) were dissected from the other hemisphere and used to measure the concentration of various A β proteins and APP forms using specific ELISAs (Johnson-Wood et al., supra).

Tissues destined for ELISAs were homogenized in 10 volumes of ice-cold guanidine buffer (5.0 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0). The homogenates
20 were mixed by gentle agitation using an Adams Nutator (Fisher) for three to four hr at RT, then stored at -20°C prior to quantitation of A β and APP. Previous experiments had shown that the analytes were stable under this storage condition, and that synthetic A β protein (Bachem) could be quantitatively recovered when spiked into homogenates of control brain tissue from mouse littermates (Johnson-Wood et al., supra).

5. Measurement of A β Levels

The brain homogenates were diluted 1:10 with ice cold Casein Diluent (0.25% casein, PBS, 0.05% sodium azide, 20 μ g/ml aprotinin, 5 mM EDTA pH 8.0, 10
30 μ g/ml leupeptin) and then centrifuged at 16,000 x g for 20 min at 4° C. The synthetic A β protein standards (1-42 amino acids) and the APP standards were prepared to include 0.5 M guanidine and 0.1% bovine serum albumin (BSA) in the final composition. The "total" A β sandwich ELISA utilizes monoclonal antibody monoclonal antibody 266, specific for amino acids 13-28 of A β (Seubert, et al.), as the capture antibody, and biotinylated

monoclonal antibody 3D6, specific for amino acids 1-5 of A β (Johnson-Wood, et al), as the reporter antibody. The 3D6 monoclonal antibody does not recognize secreted APP or full-length APP, but detects only A β species with an amino-terminal aspartic acid. This assay has a lower limit of sensitivity of ~50 ng/ml (11 nM) and shows no cross-reactivity to the endogenous murine A β protein at concentrations up to 1 ng/ml (Johnson-Wood et al., supra).

The A β 1-42 specific sandwich ELISA employs mAb 21F12, specific for amino acids 33-42 of A β (Johnson-Wood, et al.), as the capture antibody. Biotinylated mAb 3D6 is also the reporter antibody in this assay which has a lower limit of sensitivity of about 125 μ g/ml (28 μ M, Johnson-Wood et al.). For the A β ELISAs, 100 μ l of either mAb 266 (at 10 μ g/ml) or mAb 21F12 at (5 μ g/ml) was coated into the wells of 96-well immunoassay plates (Costar) by overnight incubation at RT. The solution was removed by aspiration and the wells were blocked by the addition of 200 μ l of 0.25% human serum albumin in PBS buffer for at least 1 hr at RT. Blocking solution was removed and the plates were stored desiccated at 4°C until used. The plates were rehydrated with Wash Buffer [Tris-buffered saline (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.5), plus 0.05% Tween 20] prior to use. The samples and standards were added in triplicate aliquots of 100 μ l per well and then incubated overnight at 4° C. The plates were washed at least three times with Wash Buffer between each step of the assay. The biotinylated mAb 3D6, diluted to 0.5 μ g/ml in Casein Assay Buffer (0.25% casein, PBS, 0.05% Tween 20, pH 7.4), was added and incubated in the wells for 1 hr at RT. An avidin-horseradish peroxidase conjugate, (Avidin-HRP obtained from Vector, Burlingame, CA), diluted 1:4000 in Casein Assay Buffer, was added to the wells for 1 hr at RT. The colorimetric substrate, Slow TMB-ELISA (Pierce), was added and allowed to react for 15 minutes at RT, after which the enzymatic reaction was stopped by the addition of 25 μ l 2 N H₂SO₄. The reaction product was quantified using a Molecular Devices Vmax measuring the difference in absorbance at 450 nm and 650 nm.

6. Measurement of APP Levels

Two different APP assays were utilized. The first, designated APP- α /FL, recognizes both APP-alpha (α) and full-length (FL) forms of APP. The second assay is specific for APP- α . The APP- α /FL assay recognizes secreted APP including the first 12

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5 amino acids of A β . Since the reporter antibody (2H3) is not specific to the α -clip-site, occurring between amino acids 612-613 of APP695 (Esch et al., Science 248, 1122-1124 (1990)); this assay also recognizes full length APP (APP-FL). Preliminary experiments using immobilized APP antibodies to the cytoplasmic tail of APP-FL to deplete brain
10 homogenates of APP-FL suggest that approximately 30-40% of the APP- α /FL APP is FL (data not shown). The capture antibody for both the APP- α /FL and APP- α assays is mAb 8E5, raised against amino acids 444 to 592 of the APP695 form (Games et al., supra). The reporter mAb for the APP- α /FL assay is mAb 2H3, specific for amino acids 597-608 of APP695 (Johnson-Wood et al., supra) and the reporter antibody for the APP- α assay is
15 a biotinylated derivative of mAb 16H9, raised to amino acids 605 to 611 of APP. The lower limit of sensitivity of the APP- α /FL assay is about 11 ng/ml (150 pM) (Johnson-Wood et al.) and that of the APP- α specific assay is 22 ng/ml (0.3 nM). For both APP assays, mAb 8E5 was coated onto the wells of 96-well EIA plates as described above for mAb 266. Purified, recombinant secreted APP- α was used as the reference standard for
20 the APP- α assay and the APP- α /FL assay (Esch et al., supra). The brain homogenate samples in 5 M guanidine were diluted 1:10 in ELISA Specimen Diluent (0.014 M phosphate buffer, pH 7.4, 0.6% bovine serum albumin, 0.05% thimerosal, 0.5 M NaCl, 0.1% NP40). They were then diluted 1:4 in Specimen Diluent containing 0.5 M guanidine. Diluted homogenates were then centrifuged at 16,000 x g for 15 seconds at
25 RT. The APP standards and samples were added to the plate in duplicate aliquots and incubated for 1.5 hr at RT. The biotinylated reporter antibody 2H3 or 16H9 was incubated with samples for 1 hr at RT. Streptavidin-alkaline phosphatase (Boehringer Mannheim), diluted 1:1000 in specimen diluent, was incubated in the wells for 1 hr at RT. The fluorescent substrate 4-methyl-umbellipheryl-phosphate was added for a 30-min RT incubation and the plates were read on a Cytofluor tm 2350 fluorimeter (Millipore) at 365 nm excitation and 450 nm emission.

7. Immunohistochemistry

30 Brains were fixed for three days at 4°C in 4% paraformaldehyde in PBS and then stored from one to seven days at 4°C in 1% paraformaldehyde, PBS until sectioned. Forty-micron-thick coronal sections were cut on a vibratome at RT and stored in cryoprotectant (30% glycerol, 30% ethylene glycol in phosphate buffer) at -20°C prior to immunohistochemical processing. For each brain, six sections at the level of the dorsal

hippocampus, each separated by consecutive 240 μ m intervals, were incubated overnight with one of the following antibodies: (1) a biotinylated anti-A β (mAb, 3D6, specific for human A β) diluted to a concentration of 2 μ g/ml in PBS and 1% horse serum; or (2) a biotinylated mAb specific for human APP, 8E5, diluted to a concentration of 3 μ g/ml in PBS and 1.0% horse serum; or (3) a mAb specific for glial fibrillary acidic protein (GFAP; Sigma Chemical Co.) diluted 1:500 with 0.25% Triton X-100 and 1% horse serum, in Tris-buffered saline, pH 7.4 (TBS); or (4) a mAb specific for CD11b, MAC-1 antigen, (Chemicon International) diluted 1:100 with 0.25% Triton X-100 and 1% rabbit serum in TBS; or (5) a mAb specific for MHC II antigen, (Pharmingen) diluted 1:100 with 0.25% Triton X-100 and 1% rabbit serum in TBS; or (6) a rat mAb specific for CD 43 (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS or (7) a rat mAb specific for CD 45RA (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS; or (8) a rat monoclonal A β specific for CD 45RB (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS; or (9) a rat monoclonal A β specific for CD 45 (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS; or (10) a biotinylated polyclonal hamster A β specific for CD3e (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS or (11) a rat mAb specific for CD3 (Serotec) diluted 1:200 with 1% rabbit serum in PBS; or with (12) a solution of PBS lacking a primary antibody containing 1% normal horse serum.

Sections reacted with antibody solutions listed in 1,2 and 6-12 above were pretreated with 1.0% Triton X-100, 0.4% hydrogen peroxide in PBS for 20 min at RT to block endogenous peroxidase. They were next incubated overnight at 4°C with primary antibody. Sections reacted with 3D6 or 8E5 or CD3e mAbs were then reacted for one hr at RT with a horseradish peroxidase-avidin-biotin-complex with kit components "A" and "B" diluted 1:75 in PBS (Vector Elite Standard Kit, Vector Labs, Burlingame, CA.).

Sections reacted with antibodies specific for CD 45RA, CD 45RB, CD 45, CD3 and the PBS solution devoid of primary antibody were incubated for 1 hour at RT with biotinylated anti-rat IgG (Vector) diluted 1:75 in PBS or biotinylated anti-mouse IgG (Vector) diluted 1:75 in PBS, respectively. Sections were then reacted for one hr at RT with a horseradish peroxidase-avidin-biotin-complex with kit components "A" and "B" diluted 1:75 in PBS (Vector Elite Standard Kit, Vector Labs, Burlingame, CA.).

Sections were developed in 0.01% hydrogen peroxide, 0.05% 3,3'-diaminobenzidine (DAB) at RT. Sections destined for incubation with the GFAP-, MAC-1- AND MHC II-specific antibodies were pretreated with 0.6% hydrogen peroxide at RT

to block endogenous peroxidase then incubated overnight with the primary antibody at 4°C. Sections reacted with the GFAP antibody were incubated for 1 hr at RT with biotinylated anti-mouse IgG made in horse (Vector Laboratories; Vectastain Elite ABC Kit) diluted 1:200 with TBS. The sections were next reacted for one hr with an avidin-biotin-peroxidase complex (Vector Laboratories; Vectastain Elite ABC Kit) diluted 1:1000 with TBS. Sections incubated with the MAC-1-or MHC II-specific monoclonal antibody as the primary antibody were subsequently reacted for 1 hr at RT with biotinylated anti-rat IgG made in rabbit diluted 1:200 with TBS, followed by incubation for one hr with avidin-biotin-peroxidase complex diluted 1:1000 with TBS. Sections incubated with GFAP-, MAC-1- and MHC II-specific antibodies were then visualized by treatment at RT with 0.05% DAB, 0.01% hydrogen peroxide, 0.04% nickel chloride, TBS for 4 and 11 min, respectively.

Immunolabeled sections were mounted on glass slides (VWR, Superfrost slides), air dried overnight, dipped in Propar (Anatech) and overlaid with coverslips using Permout (Fisher) as the mounting medium.

To counterstain A β plaques, a subset of the GFAP-positive sections were mounted on Superfrost slides and incubated in aqueous 1% Thioflavin S (Sigma) for 7 min following immunohistochemical processing. Sections were then dehydrated and cleared in Propar, then overlaid with coverslips mounted with Permout.

8. Image Analysis

A Videometric 150 Image Analysis System (Oncor, Inc., Gaithersburg, MD) linked to a Nikon Microphot-FX microscope through a CCD video camera and a Sony Trinitron monitor was used for quantification of the immunoreactive slides. The image of the section was stored in a video buffer and a color-and saturation-based threshold was determined to select and calculate the total pixel area occupied by the immunolabeled structures. For each section, the hippocampus was manually outlined and the total pixel area occupied by the hippocampus was calculated. The percent amyloid burden was measured as: (the fraction of the hippocampal area containing A β deposits immunoreactive with mAb 3D6) x 100. Similarly, the percent neuritic burden was measured as: (the fraction of the hippocampal area containing dystrophic neurites reactive with monoclonal antibody 8E5) x100. The C-Imaging System (Compix, Inc., Cranberry Township, PA) operating the Simple 32 Software Application program was linked to a

Nikon Microphot-FX microscope through an Optronics camera and used to quantitate the percentage of the retrosplenial cortex occupied by GFAP-positive astrocytes and MAC-1- and MHC II-positive microglia. The image of the immunoreacted section was stored in a video buffer and a monochrome-based threshold was determined to select and calculate the total pixel area occupied by immunolabeled cells. For each section, the retrosplenial cortex (RSC) was manually outlined and the total pixel area occupied by the RSC was calculated. The percent astrocytosis was defined as: (the fraction of RSC occupied by GFAP-reactive astrocytes) X 100. Similarly, percent microgliosis was defined as: (the fraction of the RSC occupied by MAC-1- or MHC II-reactive microglia) X 100. For all image analyses, six sections at the level of the dorsal hippocampus, each separated by consecutive 240 μ m intervals, were quantitated for each animal. In all cases, the treatment status of the animals was unknown to the observer.

Although the foregoing invention has been described in detail for purposes of clarity of understanding, it will be obvious that certain modifications may be practiced within the scope of the appended claims. All publications and patent documents cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each were so individually denoted.

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